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IMMUNOLOGY ABSTRACTS

Poster Sessions

Poster Session: Authophagy

P0001

Apoptotic parasites silence macrophages by misusing the autophagy machinery

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Purpose/Objective: An appropriate T cell response to *Leishmania* (Lm) infection is critical for an effective immune response. Human macrophages (MF) can present antigen to T lymphocytes and at the same time serve as host cells. Upon macrophage infection the virulent inoculum of Lm promastigotes consists of apoptotic and viable promastigotes. The viable promastigotes enter a maturing phagolysosome were they can survive and grow as amastigotes; the fate of apoptotic parasites is unclear.
Materials and methods: In this study, we hypothesize that the apoptotic promastigotes use the MFs«autophagy machinery to down regulate MF antigen presentation and T cell activation.
Results: Upon promastigote uptake by human primary MFs, we found apoptotic promastigotes to enter a compartment positive for the autophagy marker LC3. This LC3 compartment matured over time and became LAMP positive. 24 h later the compartment resolved after highly efficient parasite degradation. When co-incubated with autologous T lymphocytes, MFs infected with viable promastigotes induced a strong CD4-positive T cell proliferation. Compared to viable parasites a significantly lower T cell reactivity was observed in response to MFs inoculated with apoptotic or a mixed population of apoptotic and viable parasites. Subsequently, preliminary results suggest that only in the presence of apoptotic promastigotes and human T cells Lm infection could be sustained in human MF over a period of 7 days.
Conclusions: We found that apoptotic promastigotes enter a maturing LC3 compartment. Our data suggest that degradation of parasites in this compartment could be involved in a down regulation of T cell activation. We now further investigate and characterize the proliferating T cell subsets and how the autophagy machinery and apoptotic promastigotes may dampen immune responses in human primary macrophages.

P0002

Autophagy is activated in the B cells of patients with SLE and correlates with disease activity

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Purpose/Objective: Autophagy is increasingly appreciated as an important immune surveillance and effector mechanism, but understanding of its dynamic function in human autoimmune disease is limited. We sought to evaluate its role in the B and T lymphocytes of patients with SLE compared with healthy controls.

Materials and methods: Patient samples were collected with informed consent, and disease activity quantified by the SELENA-SLEDAI index. Multispectral imaging flow cytometry was performed using an Amnis ImageStream^X instrument. LC3-positive autophagosomes were enumerated in viable, non-apoptotic cells using the Bright Detail Intensity algorithm implemented in IDEAS 6. Autophagic flux was determined by incubation with chloroquine. As an alternative measure of autophagy, uptake of the novel autophagosomotropic dye CytoID (Enzo) was analysed using conventional flow cytometry. Autophagy was assayed in negatively selected B cells stimulated with combinations of anti-IgM and anti-CD40 antibodies, and interferon-α.
Results: Autophagy was significantly increased in the CD19$^+$ B cells of patients with SLE compared with healthy controls

($P = <0.001$, $n = 22$ patients, 15 controls), and there was a positive correlation with SLEDAI score ($r = 0.67$, $P = <0.002$). There was however, no association in CD4$^+$ T cells ($P = 0.49$). There was no statistical evidence of confounding due to patient age or medication use. Assessment of autophagic flux using the autophagosome-lysosome fusion inhibitor chloroquine revealed an accumulation of autophagsomes following treatment.

Analysis of *ex vivo* viable, annexin V negative human B cells demonstrated a significant increase in autophagy in unstimulated compared with anti-IgM stimulated cells, with further decreases observed with the addition of anti-CD40 and interferon-α.
Conclusions: The process of autophagy has not been previously examined in *ex vivo* human B cells from patients with systemic autoimmune disease. We demonstrate that autophagy is enhanced in this context. Given our *in vitro* data, we may advance the hypothesis that autophagy is acting as a survival mechanism for auto reactive B cells lacking adequate survival signals. An alternative explanation requiring further investigation is that autophagy is acting to promote presentation of self-antigens by B cells. Autophagy is readily inhibited by many common pharmaceutical agents and may therefore represent a new treatment target in SLE.

P0003

Role of autophagy in the immunopathogenesis of leprosy

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Purpose/Objective: Leprosy is a chronic infectious disease that can present different clinical forms and there is evidence that the establishment of different clinical forms is driven by host innate mechanisms. Macrophages from tuberculoid (BT) and lepromatous (LL) patients seem to have a different behavior in relation to the mycobacteria. While in LL patients there are highly infected macrophages, in BT rare or few bacilli are found. Electron microscopy studies showed the presence of phagosomes with double membrane in macrophages exposed to *M. leprae* (ML), suggesting a possible involvement of

autophagy in the immunomodulatory response. In the present study we evaluated the role of autophagy in the immune response to ML.
Materials and methods: THP-1 monocytic cell line, monocytes from healthy subjects and BT and LL macrophages isolated from skin lesions were used. For evaluate autophagy we used transmission electron microscopy, western blotting, immunohistochemistry and immunofluorescence techniques.
Results: Ultrastructural analysis showed a higher number of autophagosomes in cells from skin lesions of BT patients compared to LL patients or normal tissue. Immunoperoxidase and western blotting analysis revealed a greater tissue expression of the autophagosome marker LC3 in BT patients when compared with LL. Additionally, there was an increase on LC3-puncta expression in *ex vivo* macrophages from BT patient, in the presence or absence of IFNγ. ML stimulation induced autophagy in THP-1 macrophages. IFNγ treatment in ML-stimulated cells increased LC3-puncta expression compared with stimuli alone or non-stimulated monocytes and THP-1 macrophages. The pre-treatment with autophagic inhibitors wortmannin (Wm) or 3-methyladenine (3-MA) was able to reduce IFNγ-induced LC3 expression. IFNγ treatment promotes ML-LC3 colocalization in THP-1 macrophages. In addition, in the presence of both IFNγ and ML, there was a higher expression of Atg3. IFNγ treatment in ML-stimulated cells was able to increase IL-15 secretion in relation to non-stimulated cells, but not IL-10. Autophagic blockage by 3-MA led to decreased IL-15 levels in response to stimulation with IFNγ and ML, but did not affect the IL-10 production.
Conclusions: Together, these data indicate that in ML-stimulated macrophages, IFNγ induces the production of proinflammatory cytokines including IL-15, which contribute to host cell increase in microbicidal activity by autophagy induction. These findings may contribute to a better understanding of the mechanisms associated with leprosy immunopathogenesis.

P0004

Transient Receptor Potential Vanilloid 1 activation induces autophagy in thymocytes through ROS-regulated proteasome inhibition, UPR and AMPK/ATG4C pathways

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Purpose/Objective: Autophagy is a highly conserved process involved in lymphocyte development and differentiation. Herein, we evaluated the effects of Transient Receptor Potential Vanilloid 1 (TRPV1) activation in mouse thymocytes.
Materials and methods: Western blot and flow-cytometric analyses were performed to assess whether TRPV1 activation by 10 μM capsaicin, a specific agonist, could induce autophagy in mouse thymocytes. We used flow cytometry to investigate calcium influx and ROS generation and western blot was to elucidate the signalling pathways. Modification of CD4 and CD8 expression and DNA fragmentation were assessed by flow cytometry. Proteasome inhibition was evaluated by proteasome peptidase activity using fluorogenic peptides. UPR induction was assessed by using RT- profiler PCR array.
Results: Triggering of TRPV1 induces autophagy in mouse thymocytes. Autophagy is activated as pro-survival process since its inhibition triggered apoptotic DNA fragmentation. TRPV1 activation induced the development of a DP subpopulation expressing lower levels of both CD4 and CD8 receptors (DP^{dull}).We found that CPS induces autophagy of DP^{dull} cells, and inhibition of autophagy by 3-MA triggers apoptosis of DP^{dull} cells. Probably via the generation of oxidative stress, TRPV1 activation induces proteasome inhibition, as shown by the reduced chimotrypsin- and trypsin-like activity. Since proteasome inhibition often induces ER stress and activates the unfolded protein response (UPR) we performed a RT-PCR array in CPS-treated thymocytes to verify UPR induction. We found a significative upregulation of calreticulin and Insulin induced gene 2, that bind to misfolded proteins and prevents the export from the Endoplasmic reticulum. Moreover, both Derlin-1 and Homocysteine-inducible, ER stress-inducible, ubiquitin-like domain member 1 that are part of a complex mediating UPR, were upregulated.
Conclusions: Overall, our data indicate that TRPV1 activation in thymocytes impairs the ubiquitin-proteasome degradation pathway. As consequence, autophagy is activated in response to UPR in order to eliminate unfolded proteins and rescue cells from apoptotic death. DP^{dull} cells constitute a distinct thymocyte subpopulation involved in the responses to stress signals during thymocyte maturation, via regulating autophagy and apoptosis in a TRPV1-dependent manner.

Poster Session: Cells on the Move — Chemokines

P0005
CCX-CKR deficiency alters thymic stroma impairing thymocyte development and promoting autoimmunity

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Purpose/Objective: The ligands for the atypical chemokine receptor CCX-CKR (CCL19, CCL21 and CCL25) play an important role in the development of a self-tolerant adaptive immune system by directing migration of specific subsets of developing thymocytes to appropriate developmental niches through interactions with the chemokine receptors CCR7 and CCR9. However, despite high affinity binding of these chemokines and an apparent chemokine scavenging function, the contribution CCX-CKR makes to regulation of autoreactive T cell development remains to be clearly established.
Materials and methods: We have analysed CCX-CKR-deficient mice compared with WT C67Bl6 mice and performed detailed analysis of appearance of spontaneous peripheral autoimmune-like lesions and examined T cell development in the thymus using both immunofluorescent microscopy and flow cytometric approaches.
Results: CCX-CKR-deletion increases incidence of a spontaneous Sjögren's syndrome-like pathology, characterised by lymphocytic infiltrates in salivary glands and liver of CCX-CKR$^{-/-}$ mice, suggestive of a defect in self-tolerance when CCX-CKR is deleted. Analysis of the thymus revealed that negatively selected mature SP cells were less abundant in CCX-CKR$^{-/-}$ thymi, yet expansion of both DP and immature SP cells was apparent. Deletion of CCX-CKR profoundly reduced frequencies of DN3 thymocyte precursors and caused DN2 cells to accumulate within the medulla. These effects are likely driven by alterations in thymic stroma as CCX-CKR$^{-/-}$ mice have impaired expansion of cTECs, the cell population that expresses the highest level of CCX-CKR in the thymus. A profound decrease in CCL25 within the thymic cortex was observed in CCX-CKR$^{-/-}$ thymi, likely accounting for their defects in thymocyte distribution and frequency.
Conclusions: These findings identify a novel role for CCX-CKR in regulating cTEC expansion, which promotes optimal thymocyte development and selection important for self-tolerant adaptive immunity.

P0006
Chemokine CCL21 Expression in Macrophages is Regulated by Nuclear Factor of Activated T Cells (NFATc4) Pathways in Murine Inflammatory Arthritis

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Purpose/Objective: Nuclear factors of activated T cells (NFAT) is a family of transcription factors critical in regulating genes expression in development and disease. The pathway is an important target for cyclosporine immunosuppression in transplant rejection and in autoimmune diseases such as rheumatoid arthritis (RA). We aimed to study a functional significance and mechanisms of NFATc4 regulation of inflammatory arthritis.
Materials and methods: In genome-wide association study (GWAS), we correlated susceptibility to antibody (Ab)-mediated arthritides (serum transfer-induced arthritis and collagen Ab-induced arthritis, CAIA) of inbred murine strains with all available DNA polymorphisms/SNPs. CAIA was induced in NFATc4-ko gene deficient and in wild-type (WT) C57BL/6J mice.Differential gene expression analysis of RNA isolated from arthritic paws was performed using Affymetrix GeneChip® Mouse Gene 1.0 ST Array followed by statistical analysis, functional and unsupervised hierarchical clustering (Ingenuity Pathway Analysis, Partek).Significantly dysregulated genes were replicated using TaqMan® RT-PCR with independent RNA samples. Immunohistochemical staining of tissue sections and chemokine production in isolated macrophages were studied.
Results: Using GWAS, NFATc4 gene was discovered to be significantly associated with arthritis severity, $P < 0.000003$. Biological effect was confirmed in NFATc4-ko gene deficient mice that exhibited significantly weaker CAIA (55% downregulation, $P < 0.01$) when were compared to wild-type. The most significantly downregulated gene in paws of NFATc4-ko mice was chemokine CCL21 (11.55-fold, $P < 0.00006$).Using RT-PCR and ELISA, we showed that production of CCL21 was blocked in LPS-stimulated peritoneal macrophages from NFATc4-ko mice. Cells isolated from articular cartilage of gene-deficient mice showed significantly lower production of collagen type I and aggrecan (twofold, $P < 0.01$). LPS- and TNF-stimulated RAW-264.7 monocyte/macrophage cells produced CCL21, and chemokine expression correlated with osteoclastogenesis.
Conclusions: Transcriptome and proteome analyses confirmed *in silico* genetic association between severity of inflammatory arthritis and polymorphisms within the Nfatc4 gene. We showed that activation of macrophages is under the strong control of NFATc4 regulatory pathways.

P0007
Chemokine citrullination reduces *in vitro* and *in vivo* inflammatory activity

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Purpose/Objective: Chemokines are modified by posttranslational modifications (PTM) that may affect their biological activity. Recently we identified a novel PTM on chemokines, i.e. deimination of specific peptidylarginines to peptidylcitrullines. Moreover, protein citrullination is known to play a prominant role in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. The biological effects of this PTM were investigated on the inflammatory chemokines CXCL5, CXCL8, CXCL10, CXCL11 and CXCL12. In addition, an ELISA was developed to quantify specific citrullinated chemokines.
Materials and methods: Citrullinated arginines were identified by protein sequence analyses and mass spectrometry. Affinity of chemokines for their respective receptors and glycosaminoglycans was analysed on receptor transfectants or human leukocytes. Biological activity of citrullinated chemokines was tested *in vitro* in receptor signaling and chemotaxis assays and adhesion molecule expression was evaluated on fresh human blood. *In vivo* chemotaxis was evaluated by intraperitoneal injection of chemokines in mice and quantification of extravasated leukocytes. The influence of truncation and citrullination of CXCL8 on its angiogenic activity was tested in the cornea assay in rabbits.
Results: Human peptidylarginine deiminase (PAD) 2 and 4 modified 1 specific Arg in CXCL5, CXCL8 and CXCL10 and multiple Arg in CXCL12. Citrullination of leukocyte-derived CXCL8 depended on the presence of neutrophils. Citrullination resulted in reduced binding to glycosaminoglycans and citrullinated CXCL8 was resistant to proteolytic activation. Citrullination reduced the receptor binding properties of CXCL5, CXCL10 and CXCL12. Moreover, receptor-dependent signalling properties of CXCL5, CXCL10, CXCL11 and CXCL12 were

lower resulting in reduced neutrophil and lymhocyte chemotactic properties. Although citrullination of CXCL8 moderately reduced reduced its signaling properties, no difference in chemotactic activity was detected *in vitro*. However, upon intraperitoneal injection, both citrullinated CXCL5 and CXCL8 had a highly reduced ability to attract neutrophils to the peritoneal cavity. Citrullinated CXCL8 retained its angiogenic activity and efficiently mobilized neutrophils upon intraveneous injection.

Conclusions: PAD-dependent citrullination of chemokines dampens their inflammatory activity.

P0008

Chemokine receptor CCR7 on $CD4^+$ T cells plays a crucial role in the induction of experimental autoimmune encephalomyelitis

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Purpose/Objective: Experimental autoimmune encephalomyelitis (EAE) is the animal model for the human disease multiple sclerosis. EAE is mediated by myelin-specific $CD4^+$ helper T cells. The chemokine receptor CCR7 is an important factor for immune cell trafficking and recirculation not only in the secondary lymphoid organ, but also within target organs of an inflammatory attack. Previous data suggested that CCR7 deficiency influenced clinical disease by altering dendritic cell biology.

Materials and methods: Using different animal models for multiple sclerosis and *in vivo* imaging technique within the CNS, we investigated the role of T cells in animals which lack the CCR7 on T cells.

Results: We demonstrate here that $CD4^+$ T cell-specific constitutive deletion of CCR7 led to an impaired clinical course in EAE. In adoptive transfer EAE, mice receiving $CCR7^{-/-}$ myelin antigen T cell receptor transgenic 2d2 TH17 cells showed an earlier disease onset compared to mice adoptively transferred with $CCR7^+/^+$ 2d2 TH17 cells. Thus CCR7-deficiency on TH17 cells caused an increased encephalitogenicity in adoptive transfer EAE. We monitored the trafficking of $CCR7^{-/-}$ and $CCR7^+/^+$ 2d2 Th17 cells within the CNS by two-photon laser scanning microscopy in living anaesthetized mice and identified distinct motility patterns. In contrast to the findings in adoptive transfer EAE, CCR7 deficiency on $CD4^+$ cells led to a delayed disease onset in active EAE induced with MOG in lymphopenic $RAG^{-/-}$ mice, which had been grafted with $CCR7^{-/-}$, $CCR7^+/^+$ or $CCR7^+/^-$. This could be attributed to impaired T cell priming in secondary lymphoid organs, which most likely resulted from reduced lymph node homing potential.

Conclusions: Taken together these findings underline a crucial and paradoxic role of CCR7 in neuroinflammation.

P0009

Chemokine-mediated innate immune responses to mosquito bites and their viruses

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Purpose/Objective: Diseases spread by mosquitoes have a significant impact on human health and include disease caused by viruses such as Chikungunya and West Nile virus. There remains a need to understand the complex interplay between these viruses, their mosquito vectors and their mammalian hosts.The early events of infection are critically important for disease outcome in the host. Strikingly, arbovirus inoculated via bites or accompanied by arthropod saliva induces a more rapid viraemia, higher mortality and higher pathogen load, compared to inoculation via a needle. Although, how virus disseminates from the inoculation site and what role arthropod saliva have in facilitating this process are not understood.

Chemokines have pivotal functions in the immune system, without which coordinated immune responses would not occur. We have studied chemotactic responses during key stages of infection, from the mosquito bite site to target CNS tissue.

Materials and methods: We have used a relevant, tractable *in vivo* model system to study the effects of bites and arbovirus infection on the chemokine system and other key innate immune processes.We have carefully mapped the kinetics of virus spread from skin inoculation sites and assayed the expression of chemokines and innate immune genes during these early stages of infection.

Results: Surprisingly, virus spreads within hours from skin to draining lymph nodes followed by viraemia at 48 h, suggesting that virus primarily disseminates away from bite sites via lymph fluid.Mosquito bites trigger chemotactic responses in the skin and lymph nodes that are qualitatively and temporally distinct from those triggered by virus infection. Bites induce a chemokine response dominated by neutrophil attracting chemokines whilst virus infection induces a later response dominated by the ligands for CXCR3.

Conclusions: In summary, we have developed a new model for studying chemotactic processes *in vivo* and defined a key aspect of innate anti-viral immunity in the initiating stages of a mosquito-borne virus infection.

P0010

Cloning of atypical chemokine receptors CRAM-A and CRAM-B for comparative functional analysis

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Purpose/Objective: Atypical chemokine receptors play an important role in the termination of inflammatory responses. Upon binding to their cognate ligands, chemokine gradient drops and immune cell migration is hampered. CCRL2 is the newest member of atypical chemokine receptor family. The human CCRL2 gene has two transcript variants CRAM-A (Chemokine Receptor on Activated Macrophages) and CRAM-B. The aim of this study is to compare the effect of these different variants on the chemokine gradient.

Materials and methods: Total cDNA was synthesized using RT-PCR. CRAM-A and CRAM-B protein coding cDNAs, specific primers and *Pfu* DNA polymerase were used in amplification and site-directed cloning (*Nhe*I and *Xma*I restriction enzyme sites). CRAM-A and -B amplicons, pIRES2-EGFP and pcDNA3.1/CT-GFP eukaryotic expression vectors were digested, isolated, ligated, and then transformed into *E.coli* competent bacteria.

Results: The resulting constructs were confirmed by restriction analysis and DNA sequencing.The recombinant clones pCRAM-A-IRES2-EGFP and pCRAM-B-IRES2-EGFP enabled the bicistronic expression of CRAM and EGFP, whereas pcDNA3.1/CRAM-A-CT-GFP and pcDNA3.1/CRAM-B-CT-GFP produced a hybrid GFP-tagged protein. The recombinant plasmids and empty vectors were delivered into HEK293T cell line via liposomal transfection. Transfection efficiency (GFP expression) and recombinant CRAM expression were analyzed by flow cytometry.

Conclusions: In conclusion, the transcript variants of human CCRL2 gene were cloned into different recombinant DNA constructs and *de novo* expression of recombinant CRAM proteins were determined. Next, the ligand binding assays will be performed with CRAM-A or CRAM-B-expressing cells.

P0011

Combining classical and NKT-cell-mediated DC licensing not only enhances, but also accelerates CD8$^+$ T cell responses

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Purpose/Objective: Dendritic cell (DC) licensing is a requisite for the induction of efficient CD8$^+$ T cell responses to extracellular antigens. Classically, DCs are licensed by CD4$^+$ helper cells. As an alternative, NKT cells can replace CD4$^+$ help. We have shown previously that combining both licensing systems results in synergistically enhanced CD8$^+$ T cell responses, by mechanisms involving the chemokine receptors CCR4 and CCR5. Here we asked whether this chemokine synergism impacts the kinetics of CD8$^+$ T cell induction.
Materials and methods: We performed *in vivo* kinetic studies using the OT-I mouse model, as well as an endogenous T cell repertoire, to analyse the initiation of CD8$^+$ T cell activation and the clearance of an adenoviral infection. Classical DC licensing was initiated by the application of the TLR-9-ligand CpG, whereas NKT-cell-mediated DC licensing was induced by the NKT-cell-ligand α-Galactosylceramide (GC).
Results: After applying the model antigen Ovalbumin (OVA) together with both CpG and GC, specific CD8$^+$ T cells proliferated faster and produced earlier more cytokines than when only one licensing system was engaged. The combination of OVA$^+$GC$^+$CpG resulted in higher antigen-specific cytotoxicity *in vivo* at earlier time points and a faster clearance of adenoviral infection in contrast to mice treated with OVA plus either adjuvant alone. This acceleration was chemokine receptor-dependent.
Conclusions: Combining classical and NKT-cell-mediated DC licensing allowed faster activation of CD8$^+$ T cells and accelerated clearance of adenoviral infection. These findings provide a novel approach for designing better vaccines against rapidly proliferating microbes.

P0012

Conditional knockout of chemokine receptor CXCR5 in antigen presenting (CD11c$^+$) cells

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Purpose/Objective: The aim of this study was to generate a conditional CXCR5 knockout transgenic mouse model using the Cre/LoxP system. Using this model we can delete CXCR5 from various cells of the immune system, preventing their localising to lymphoid follicles. These models can then be used to study the possible loss of function of specific follicular localising cells.
Materials and methods: LoxP sites were gene-targeted to surround the CXCR5 gene in C57Bl/6 mice. To assess the efficacy of this model we have introduced the CD19-Cre(1) transgene to delete CXCR5 from B-cells. To study the role of antigen-presenting cells we have combined the CXCR5 floxed allele with CD11c-Cre(2) transgenic mice.
Results: We have generated and are currently characterising the CXCR5 floxed transgenic mouse model. Following CD11c-Cre mediated recombination we observed no alteration in lymphoid organogenesis, suggesting that removal of CXCR5 expression by potential CD11c$^+$ lymphoid tissue inducer cells had no effect on their ability to promote or maintain development of lymphoid organs including spleen, lymph nodes, intestinal Peyer's patches or isolated lymphoid follicles. Investigation of lymphoid microarchitecture revealed retention of CD11c$^+$ve cells within T-cell areas.
Conclusions: Generation of CD11c-restricted CXCR5 knockout had no effect on lymphoid organogenesis but has impaired the ability of CD11c$^+$ cells from entering the B-cell follicle. These cells have recently been reported to regulate the development of T_H2 responses within this locale (3) using complex mixed bone marrow chimeric and cell deletion based models. We present here a refined genetically-regulated cell-specific model to study the role of antigen presenting cells in lymphoid follicles and data on effects upon immune function.

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P0013

Inducible tertiary lymphoid structures formation in a novel model of sialoadenitis in wild-type mice is preceded by ectopic expression of lymphoid chemokines

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Purpose/Objective: Salivary glands of patients with Sjögren's syndrome (SS) develop ectopic lymphoid structures (ELS) characterized by B/T cell compartmentalization, the formation of high endothelial venules (HEV), follicular dendritic cell networks (FDCs), functional B cell activation with expression of activation-induced cytidine deaminase (AID) as well as local differentiation of autoreactive plasma cells. The mechanisms triggering ELS formation, autoimmunity and exocrine dysfunction in SS are largely unknown. Here we present a novel model of inducible ectopic lymphoid tissue formation, breach of humoral self-tolerance and salivary hypofunction following delivery of a replication-deficient adenovirus-5 (AdV5) in submandibular glands of C57BL/6 mice through retrograde excretory duct cannulation.
Materials and methods: Luciferase- or LacZ-encoding Ad5 were delivered in C57BL/6 mice salivary glands (SG) through retrograde cannulation. SGs were collected at various time-points 1, 2 and 3 weeks post-cannulation (pc) and frozen sections were graded for infiltration and stained for T/B cell segregation, FDCs and HEV markers. Submandibular salivary flow was induced by pilocarpine stimulation and the amount of saliva measured. Expression of TLS-related genes was investigated by TaqMan-PCR. Anti-viral antibodies and autoantibodies were detected by IF and western blot.
Results: In this model, inflammation rapidly and consistently evolves from diffuse infiltration towards the development of SS-like periductal lymphoid aggregates within 2 weeks from AdV delivery. These infiltrates progressively acquire ELS features and support functional GL7$^+$ /AID$^+$ germinal centers. Formation of ELS is preceded by ectopic expression of lymphoid chemokines CXCL13, CCL21 and CCL19 as well as lymphotoxin-β and is associated with development of antinuclear antibodies in 75% of the mice. Finally, reduction in salivary flow was observed over 3 weeks post AdV infection consistent with exocrine gland dysfunction as a consequence of the inflammatory response.
Conclusions: This novel model has the potential to unravel the cellular and molecular mechanisms regulating ELS formation and their role in exocrine dysfunction and autoimmunity in SS.

P0015

Influence of placental secretory factors to endothelial cell functional activity and monocyte-endothelial interaction

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Purpose/Objective: Such complication of pregnancy as preeclampsia is accompanied by trophoblast differentiation and placental angiogenesis disruption and local placental inflammation. Macrophages and endothelial cells are the main source of cytokines and angiogenic factors in placenta. Those factors have an autocrine and paracrine effect. The goal of this investigation was to evaluate the influence of placental secretory factors to endothelial cells (ECs) functional activity and monocyte-endothelial interaction.

Materials and methods: Placental tissue samples were taken from healthy pregnant women on 9−11 weeks (abortion) and 38−39 weeks of gestation, pregnant women with gestosis on 38−39 weeks of gestation (both after Cesarean section) and were incubated in DMEM/F12, 10% FBS. The ECs line EA.hy926 was grown in DMEM/F12, 10% FBS. Monocyte cell line THP-1 was grown in RPMI-1640 medium, 10% FBS. ECs were incubated with placental supernatants to characterize its influence to ECs phenotype and activity.

Results: Angiogenic factors secretion was decreased in third trimester in comparison with first trimester normal pregnancy; preeclamptic pregnancy is characterized with increased placental secretion of antiangiogenic factors in comparison with normal pregnancy. Third trimester placental supernatants caused a decrease of ECs angiogenic receptors and adhesion molecules expression, ECs proliferation and migration, monocyte transendothelial migration in comparison to first trimester placental supernatants. It can be turned to placental growth limitation to the end of pregnancy. Preeclamptic placental supernatants caused increase of CD119 ECs expression and bFGF ECs secretion, decrease of ECs proliferation and migration in comparison to normal pregnancy supernatants. Monocyte transendothelial migration activity was higher to preeclamptic placental supernatants in comparison to normal pregnancy one and was accompanied with increased monocyte CD11b expression.

Conclusions: Discovered ECs and monocyte cells phenotype and functional alterations can contribute to angiogenic abnormalities and local placental inflammation initiation during preeclamptic pregnancy.

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P0016

Inhibition of neutrophil chemokine receptor CXCR2 profoundly suppresses inflammation-driven and spontaneous tumorigenesis

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Purpose/Objective: Certain properties of the chemokine receptor CXCR2 suggest pro-tumorigenic potential: others, such as reinforcing senescence, could block tumor progression.However, it is unclear whether CXCR2 plays any indispensable role in *de novo* tumorigenesis *in vivo*.To definitively address this, we investigated CXCR2 function in inflammation-driven and spontaneous mouse models of intestinal and skin neoplasia, including benign and malignant tumor models.

Materials and methods: Skin inflammation was induced by applying TPA (Sigma) to shaved dorsal skin. Skin papillomas were induced with a single application of 25 mg of DMBA (Sigma) followed by tri-weekly TPA treatments for up to 22 weeks.Colitis was induced by providing *ad libitum* with 2% or 3.5% (w/v) DSS (MP Biomedicals, MW 36−50 kDa) in place ofdrinking water for 5 days.

Transcripts were measured by Q-RT-PCR on a 7900HT Fast-Real Time PCR System (Applied Biosystems), using TaqMan gene expression assays (Applied Biosystems)

For pepducin treatments, $Apc^{Min/+}$ mice were injected s.c. with 2.5 mg/kg × 1/2pal-i3 pepducin [Genscript, RTLFKAHMGQKHR, palmitoyl N-terminal, amidation C-terminal (21)] or control × 1/2pal-i3CONT pepducin in sterile saline, and then daily with 1 mg/kg.For neutrophil depletion, 35 day-old $Apc^{Min/+}$ mice were injected i.p. 3× a week with 200 mg of 1A8 or 2A3 isotype control 3× weekly.

Results: All tumors developing in these models had substantially elevated expression of chemokine ligands for CXCR2. Moreover, CXCR2 deficiency profoundly suppressed inflammation-driven tumorigenesis in skin and intestine and spontaneous adenocarcinoma formation in *AhCreER* $APC^{fl/+}$ $PTEN^{fl/fl}$ mice, while 'pepducin'-mediated CXCR2 inhibition reduced tumorigenesis in $Apc^{Min/+}$ mice. Established tumors contained CXCR2-dependent neutrophil infiltrates. CXCR2 deficiency dramatically attenuated neutrophil recruitment, and concomitant epithelial proliferation, during tumor-promoting inflammation.Moreover, neutrophil depletion suppressed growth of established skin tumors and reduced adenoma formation in $Apc^{Min/+}$ mice.

Conclusions: CXCR2 is a potent pro-tumorigenic chemokine receptor in skin and intestine due, at least in part, to its ability to direct neutrophil recruitment during tumor-inducing and tumor-driven inflammation.Our data suggest that CXCR2 antagonists may have utility as cancer therapeutics, or as prophylactics in patients at high risk of colorectal cancer.

P0017

Inhibition of neutrophils recruitment to the lung in the course of pneumonic plague is associated with suppression of pulmonary KC, MIP-2 and G-CSF production

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Purpose/Objective: Bacterial infection of the lung triggers a prompt innate immune response mainly characterized by infiltration of neutrophils into the infected tissue. Recruitment of neutrophils from the blood to the site of infection is mediated by several key effector molecules, including KC (CXCL1), MIP-2 (CXCL2) and G-CSF. These soluble factors are locally produced in the lung and diffuse to the blood ensuring effective neutrophil migration and function upon demand. Pneumonic plague, initiated by *Yersinia pestis* (*Y. pestis*) airway-infection, is a fatal and rapidly progressing disease. The pathogen replicates initially in the lung, causing substantial damage to the tissue followed by dissemination to internal organs and blood. In this study we monitored neutrophil recruitment to the lung concomitant with cytokines production, in the course of pneumonic plague.

Materials and methods: C57BL/6 mice were intranasally inoculated with a lethal dose of the virulent *Y. pestis* Kimberley53 strain (100LD50). Disease progression was monitored at various time points post infection (p.i.).

Results: Infected mice exhibit attenuated neutrophil infiltration from the blood to the lung in the first 24 h p.i. Accordingly, levels of KC, MIP-2 and G-CSF were not elevated in the blood at this early time point. However, at a late stage of disease progression − 48 h p.i., a

significant elevation of KC, MIP-2 and G-CSF levels in the blood and lung was accompanied by neutrophil accumulation in the lung. By using a trans-well migration assay, we evaluated whole-lung supernatants obtained from infected mice, for their potential to support neutrophil migration. Only supernatants from mice which were sacrificed 48 h p.i. indeed augmented neutrophils trans-well migration. Real-time PCR analysis of whole-lung mRNA revealed delayed expression of KC, MIP-2 and G-CSF at 48 h p.i. In contrast, infection with an avirulent strain was characterized by immediate up-regulation of KC, MIP-2 and G-CSF mRNA, neutrophils infiltration and inhibition of bacterial proliferation and dissemination.
Conclusions: The data suggests that during early stages of pneumonic plague neutrophil chemoattractans expression is suppressed therefore leading to an ineffective immune response against the *Y. pestis* pathogen.

P0018
Investigation of signaling mediators which control bone marrow memory T cell migration

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Purpose/Objective: We have previously identified a population of memory $CD4^+$ T cells which preferentially reside in the bone marrow of mice and can be classified as resting in terms of their proliferation state and major transcriptional activity. However, after activation by antigen, these cells are extremely capable cytokine producers and support the production of high affinity antibodies, proving that they are highly effective memory cells. The major objective of this study is to investigate whether such memory cells in the bone marrow of mice and humans can be further classified as resting in terms of their migratory capacity, and if so, what are the molecular mechanisms which mediate this phenotype.
Materials and methods: Chemokine receptor expression on memory ($CD44^{hi}$) CD4 and CD8 T cells from bone marrow and spleens of mice were assessed by flow cytometry. *In vitro* migratory potential was assessed using transwell migration assays. Migration was also examined in more detail using confocal microscopy based methods (3D chemotaxis slide) and polarisation assays. For analysis of signaling molecules involved in mediating migration of memory T cells, global transcriptional microarray profiles were analysed and genes related to migration were grouped using gene set enrichment analysis.
Results: Murine memory bone marrow T cells were found to exhibit differential chemokine receptor expression, as assessed by transcriptome analysis and on the protein level by flow cytometry. In particular, CCR2 and CXCR6 levels were increased on bone marrow memory T cells. *In vitro* transwell migration assays revealed that bone marrow T cells migrated less than splenic T cells when no stimulus was added to the chambers. However, when the cells were exposed to chemokines, the bone marrow counterparts were found to be more responsive to stimuli such as CXCL12 than splenic T cells.
Conclusions: Bone marrow memory T cells differ from splenic memory T cells in terms of their chemokine receptor expression and migration patterns. A variety of candidate molecules are currently being tested to identify their roles in the control of bone marrow memory T cell migration.

P0019
Macrophage migration inhibitory factor regulates neutrophil chemotactic responses in bacterial infection

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Purpose/Objective: Polymorphonuclear neutrophils mainly facilitates the defense against invading uropathogenic *E.coli* (UPECs) within the urinary tract and are rapidly recruited into the infected urinary bladder. Although the macrophage inhibitory protein-2 (MIP-2) is considered the major chemokine responsible for this recruitment, the role of other chemokines involved in this recruitment are currently unknown.
Materials and methods: Wild type (C57/B6) and CCR5 knockout mice were infected 3h h before analysis with UPECs. If indicated MIF, KC and MIP-2 were blocked before using 125 ng blocking antibody per gram mouse weight. Cells were isolated from the bladder tissue by collagenase/DNAse digestion and analyzed by flow cytometry.
Results: We could show that the initial recruitment of neutrophils into the *E.coli*-infected murine bladder is induced by tissue resident macrophages. Such macrophages produced the CXCR2 ligands MIP-2, MIF and KC. Blockade of the neutrophil chemokine KC, MIP-2 and the macrophage inhibitory factor (MIF) demonstrated, that KC and MIF, but not MIP-2 mainly reduced neutrophil infiltration into the bladder. Apart from these CXCR2-ligands also CCR5 seems to contribute to neutrophil recruitment into the inflamed bladder as the number of neutrophils was decreased in CCR5-deficient animals. Of note, chemokines that recruit neutrophils did not influence the number of inflammatory $Gr1^+$ macrophages, which shows the specificity of these chemokines for neutrophils and suggests that the subsequent recruitment of inflammatory macrophages are independent of neutrophils.
Conclusions: These data demonstrates that a plethora of different chemokines specifically regulate the recruitment of neutrophils into the infected bladder and might be crucial for the innate immune repsonse against UPECs.

P0020
Myosin Vb mobilizes the chemokine decoy receptor D6 from recycling endosome for adaptive upregulation and chemokine degradation

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Purpose/Objective: The decoy receptor D6 is a chemokine scavenger with a non-redundant role in the control of inflammation. D6 degradative properties refer to as ligand-induced increase of receptor membrane levels through a rapid mobilization from recycling endosomes but the mechanisms by which this adaptive up-regulation is achieved are still undefined. Given the importance of cytoskeleton dynamics in regulating chemokine receptors intracellular trafficking, here we investigate the role of actin and microtubules on D6 trafficking and scavenging properties.
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Materials and methods: Confocal microscopy analysis has been used to evaluate D6 intracellular localization and actin/microtubule organization, using depolymerizing and stabilizing agents of actin filaments and microtubules. D6 membrane expression was measured with FACS, and chemokine degradation by ELISA test.
Results: In basal conditions D6 colocalizes with both microtubules and myosin Vb but not actin filaments. Perturbations of microtubule dynamics do not affect D6 constitutive endocytosis, however disrup-

tion of recycling endosomes by nocodazole increases D6 surface levels and this effect is reverted by dominant negative Rab11, indicating that intact microtubule network is required for the correct sorting of D6 to slow recycling endosomes. On the contrary, inhibition of actin turnover increases D6 expression on cell surface as a consequence of impaired receptor constitutive endocytosis. Interestingly, the actin depolymerization agent latrunculin A increases D6 membrane expression without modifying receptor internalization rate and this effect is reverted by dominant negative Rab4, indicating that D6 is missorted to rapid recycling endosomes. After chemokine exposure, actin fibres and microtubules rearrange at plasma membrane where strongly colocalized with D6-myosin Vb complexes. Microtubules depolymerization and myosin Vb inactivation impair D6 up-regulation and scavenging, suggesting that intact microtubules and myosin Vb are required to mobilize D6 from recycling endosomes.

Conclusions: Cytoskeletal dynamics sustain D6 constitutive endocytosis and its correct sorting to Rab4/Rab11 recycling endosome, and myosinVb-dependent control of cytoskeletal rearrangement is required for the adaptive up-regulation of D6 allowing optimal degradation of chemokines.

P0021
Peripheral monocyte expression of CCR2 is altered by pregnancy

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Purpose/Objective: Purpose: Peripheral monocytes are chemotactically recruited to the decidua during pregnancy where they differentiate into macrophages clearing cellular debris and facilitating trophoblast invasion. Previous studies suggest that monocyte phenotype, behaviour and activation are affected by pregnancy. The purpose of this study was to investigate how pregnancy affects monocyte surface expression of CCR2, PBMC-secretion of the CCR2 ligand CCL2/MCP-1 and migratory behaviour of monocytes. In addition, we investigated placental expression of CCR2 and how *in vivo* CCL2/MCP-1 concentrations vary during and after pregnancy.

Materials and methods: Material and methods: PBMCs from women at labour and non-pregnant women were cultured *in vitro*. Monocyte surface markers were evaluated with flow cytometry and transwell-experiments were performed to study monocyte migratory response to CCL2/MCP-1. Placental expression of CCR2 was performed using immunohistochemistry and CCL2/MCP-1-concentrations in supernatants and serum were measured with CBA.

Results: Results: This study demonstrates that women at labour have significantly reduced percentages of CD16-CCR2$^+$ and CD16$^+$ CCR2$^+$ monocytes compared to non-pregnant women. Further, we show that CD16- and CD16$^+$ monocytes from women at labour and non-pregnant women have a similar migratory capacity towards CCL2/MCP-1 *in vitro*. In addition, CCR2$^+$ cells were abundant in the deciduas of all placentas. Finally, we found that PBMCs from women at labour release more CCL2/MCP-1 compared to non-pregnant women, while the *in vivo* concentrations of CCL2/MCP-1 was lower during pregnancy compared to after pregnancy.

Conclusions: Conclusions: We demonstrate that pregnancy significantly reduces the percentage of CCR2$^+$ monocytes in both CD16- and CD16$^+$ subpopulations, which might be explained by migration towards the decidua during pregnancy. Indeed, the monocytes from pregnant women readily migrated towards CCL2/MCP-1 *in vitro* and a strong expression of CCR2 was found on cells in the placental decidua. Uncontrolled placental infiltration of monocytes has been associated with pregnancy complications and our results suggest that monocyte migration is tightly regulated during pregnancy.

P0022
The biochemical properties of the D6 N-terminus and its therapeutic potential

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Purpose/Objective: D6 is an 'atypical chemokine receptor' and the N-terminal region (D6-N) is thought to act as part of the binding site for inflammatory chemokine ligands. A synthetic peptide analogue of D6-N may therefore have the capability to bind a broad spectrum of inflammatory chemokines, and consequently inhibit receptor binding. Thus D6-N may have the potential to be used therapeutically as a non-immunogenic, broad-based chemokine scavenger that would be beneficial in the treatment of chronic inflammatory conditions. Binding-affinity studies using different experimental techniques suggest a strong interaction between D6-N and different inflammatory chemokines.

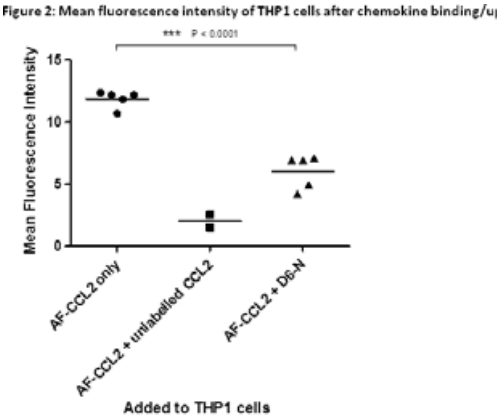
Materials and methods: A chemically synthesised version of the N-terminal of D6 (D6-N) has been used in these experiments.

Assessment of the ability of D6-N to bind inflammatory CC chemokines: Mixtures of biotinylated and native chemokines were incubated with D6-N and streptavidin beads used to 'pull-down' the chemokines. 'Pull-down' samples were then run on SDS gels and western blotted to examine any co-pull down of D6-N.

Assessing the ability of D6-N to interfere with CCL2/CCR2 interactions: CCL2 labelled with Alexafluor-647 (AF-CCL2) was added to THP1 cells with either unlabelled CCL2 or D6-N (as binding competitors) for 1 h. Cells were then analysed by FACS to measure CCL2 binding and uptake.

Results: i) D6-N binding properties: Bands corresponding to D6-N were apparent on western blots when CCL2 and CCL22 were used to 'pull down' D6-N. There was virtually no D6-N detected in samples when the non-D6 ligand, CCL19 was used therefore confirming specificity of D6-N for inflammatory chemokines.

ii) D6-N and CCL2/CCR2 binding: Addition of AF-CCL2 to THP1 cells resulted in an increase in the mean fluorescence intensity associated (MFI) with AF-CCL2 binding and uptake by the cells. As expected, this could be 'competed out' by adding a 20-fold molar excess of un-labelled CCL2. Importantly, the addition of D6-N also significantly reduces MFI associated with AF-CCL2 binding to, and internalisation by, the THP1 cells.



Chemokine(s) used	Relative density of D6-N band on western blot
Negative control	1
Biotinylated CCL2	10.42
Biotinylated CCL2$^+$ CCL22	36.09
Biotinylated CCL19	1.84
Biotinylated CCL19$^+$ CCL22	32.85
CCL22	31.05

Conclusions: The D6-N peptide is capable of binding to the inflammatory chemokines CCL2 and CCL22 but not to the homeostatic chemokine CCL19.

Addition of D6-N inhibits AF-CCL2 uptake by CCR2 on THP1 cells, suggesting that D6-N can bind to AF-CCL2, and thus prevent it from binding to its receptor.

P0023

The chemokine receptor CX3CR1 mediates homeostatic DC-colonization of the kidneys and leukocyte infiltration in Nephrotoxic Nephritis

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Purpose/Objective: Dendritic Cells (DCs) span the tubulointerstitium of the kidneys and monitor the renal parenchyma. Although renal DCs (rDCs) form a contiguous network, the anatomy of the kidney suggests that DCs located in the renal cortex, where most food antigens are reabsorbed, will differ from DCs located in the hyperosmotic and hypoxic medulla. Here, we investigated phenotypical and functional differences between cortical and medullary DCs, as well as the role of the chemokine receptor CX3CR1, which is expressed by most rDCs, in homeostasis and Nephrotoxic Nephritis (NTN).
Materials and methods: Renal cortex and medulla were macroscopically separated prior to DC isolation. DC phenotype and function were investigated by flow cytometry and functional assays. To study rDCs under inflammatory conditions, NTN, in which rDCs are protective at the onset of disease but are rendered pathogenic by the inflammatory environment induced by macrophage driven DTH responses, was induced. The role of CX3CR1 in NTN was investigated, using CX3CR1$^{GFP/GPF}$ transgenic mice.
Results: Under homeostatic conditions, DCs were more abundant in the renal cortex as compared to the medulla. Medullary DCs took up antigen more efficiently than cortical DCs. However, expression of costimulatory markers and the ability to stimulate T cells were similarly low in DCs isolated from either compartment.

Upon NTN induction, antigen uptake increased in both rDC subsets. Nevertheless, only cortical, but not medullary, DCs became competent to drive CD4 T cell proliferation and production of IFN-γ and IL-17.

In CX3CR1$^{GFP/GPF}$ transgenic mice, we discovered a reduction of rDCs under homeostatic conditions, which was more pronounced in the cortex. Interestingly, in these mice, NTN was attenuated as compared to control mice, as shown by histological and functional parameters. Flow cytometric analysis of the kidneys after NTN induction revealed reduced infiltration of DCs and macrophages in CX3CR1$^{GFP/GPF}$ transgenic mice as compared to heterozygous controls.
Conclusions: These findings suggest that cortical DCs may be more immunostimulatory as compared to medullary DCs, and drive Th1 as well as Th17 responses in NTN. Furthermore, CX3CR1 seems to play a role in DC-colonization of the homeostatic kidneys, especially in the cortical compartment, as well as in the recruitment of pro-inflammatory cells in NTN.

P0024

The microRNA miR-223 controls pulmonary trafficking of leukocytes in tuberculosis by targeting innate cytokines

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Purpose/Objective: Tissue recruitment of immune cells during infection is critical for efficient eradication of pathogens. Yet, excessive accumulation of inflammatory cells is deleterious due to subsequently induced tissue damage. Recruitment of leukocytes at infection site is tightly regulated by chemoattractants. While regulation of chemoattractants by cellular and soluble immune mediators was described, a role for micro RNAs (miRs) was sparsely studied so far. We aimed at understanding how miRs regulate trafficking of immune cells in tuberculosis (TB), a chronic, non-resolving inflammation.
Materials and methods: Lung specimens from TB patients and experimentally infected mice were profiled for miRs expression using microarrays and subsequently miR candidates were confirmed by qPCR. Relevance of selected miRs in TB control was further tested by analysing responses to infection in gene knock-out mice. MiR targets and their effects on inflammation, lung cell trafficking and disease outcome were evaluated by combining *in silico* approaches with molecular and cellular investigations.
Results: We identified microRNA-223 (miR-223) as one of the most abundant noncoding RNAs in lung biopsies of TB patients. A similar pattern of expression was detected in TB-susceptible mice. Deletion of miR-223 rendered resistant mice highly susceptible to TB due to aberrant neutrophil-driven inflammation. MiR-223 tailored lung recruitment of innate cells by targeting the chemokines CXCL2, CCL3 and the cytokine IL-6.
Conclusions: Our study demonstrates that miR-223 is an essential regulator of inflammation in TB. By identifying innate cytokines as novel targets, we ascribe new biological functions to miR-223, namely control of leukocyte chemotaxis. The miR-dependent regulation of cell trafficking could be a general mechanism in inflammatory diseases.

P0025

The role of the atypical chemokine receptor CCRL2 (CRAM) in shaping local chemokine gradients

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Purpose/Objective: The correct localistation of immune cells during development, homeostasis and pathogenesis is an important prerequesite for the correct function of the immune system. The temporal and spatial control of this localisation is achieved by a complex and tightly regulated network of signalling molecules, among which chemokines play a prominent role. Besides the classical chemokine receptors, atypical, non-signalling receptors have been found to be important for the finetuning of the chemokine network by regulating

chemokine availability as well as interfering with the signaling of classical receptors. The most recently identified member of this atypical family is CCRL2, also called CRAM. We have shown in previous work that CCRL2 binds the homeostatic chemokine CCL19, which plays a pivotal role in the development and function of B and T cells through its classical receptor CCR7. When coexpressed with CCR7, CRAM interferes with the classical CCR7 responses.

Materials and methods: We used in-vitro transwell systems to simulate migration and transmigration of lymphocytes in response to chemokine gradients, as well as cell-cell adhesion assay. Furthermore, the subcellular localisation of CRAM was analysed by immune fluorescence (IF) and confocal microscopy. CRAM expression in human tissue sections was analysed by immunohistchemistry.

Results: We demonstrate that CRAM is able to efficiently internalise and re-express its ligand CCL19, without inducing significant degradation. CRAM expressing cells can subsequently present CCL19 to CCR7 expressing cells and induce classical responses such as calcium mobilisation and chemotaxis as efficiently as direct application of the chemokine. Furthermore, CRAM was shown to be involved in the transport of CCL19 across a polarised cell monolayer.

Conclusions: Our results suggest a role for CRAM in the finetuning of CCL19 gradients. By internalising, transporting and presenting CCL19, CRAM expressing cells locally enrich CCL19, thus creating gradients and ensuring the attraction of CCR7 expressing cells.

P0026

TNFa modulates chemokine expression and leukocyte influx during alveolar bone repair process in mice

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Purpose/Objective: Although the migration of innate immunity related leukocyte subsets, especially macrophages, have been implicated in bone repair process, the molecular mechanisms underlying such process remains unknown. Thus the aim of this study was to characterize the role of TNF-α (pro-inflammatory cytokine) in the modulation of chemokine expression and in leukocyte influx in alveolar bone repair process after tooth extraction in mice.

Materials and methods: C57Bl/6 (WT) and TNFp55KO strains had the right upper incisor was extracted and the maxilla containing the remaining alveolus was collected 0, 7, 14, 21, 28 and 42 days after extraction for molecular (RealTimePCR) and histomorphometric analysis.

Results: The results demonstrated that in C57Bl/6WT mice the expression of CCL2, CCL25 and CX3CL1 was associated with the development of the inflammatory infiltrate and a granulation tissue; TNF-α expression peaked at 7 days while the chemokines peaked with 14 days. Besides, growth factors as BMP2, BMP4, BMP7, TGFb1 and VEGFa had a gradual increase reaching its peak with 7 days. Afterward the granulation tissue was gradually (7−42 days) replaced by connective tissue, blood vessels and bone. These processes were associated with higher expression of matrix markers (COL1a1, COL1a2, COL2a1, MMP1α) and bone markers RUNX2 (CBFA-1) and ALP, and subsequently by OCN, DMP1 and PHEX), concomitant with lower expression of AHSG and AMBN. Subsequently, bone remodeling features were detectable, in parallel with increased expression of osteocyte marker SOST. TNFp55KO strain presented an increased number of leukocytes in repair sites despite a slight decrease in CXCL1, CCL-2 and CCL3 expression. TNFp55KO also presented a delay in angiogenesis and in transition of granulation to bone tissue, a discreet presence of newly formed bone tissue associated with decreased CBFA-1, ALP, OCN and PHEX expression, in opposite of the higher proportion of osteoclasts in the late periods of the repair process.

Conclusions: The results presented demonstrate that the absence of TNF-alpha interferes in alveolar bone repair through mechanisms that involve the modulation of osteogenic markers and chemokines expression, as well the modulation of inflammatory cell migration along the course of alveolar bone repair.

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Poster Session: Complement

P0027

Atypical Hémolytique and Uremique Syndrom by MCP defenciency diagnosed at a teenager

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Purpose/Objective: The relation between the atypical H*mlytique-Uremiquse Syndrom and the deficits in fractions of complement.
Materials and methods: We report the observation of a 17-yaer-old teenager, whose first symptom appeared in january 2011.She has brutally, without a triggering factor, presented anaemia, a severe hypertention and anuric acute renal failure.On the biological plan, we are noticed:hemlytic anaemia with scizocytes, very high LDL and rate of haptoglobin collapsed, the studdy on the activity of the complement was realized and it objectified a deficit in MCP (CD46) with factor H and I normal. A transjugulaire renal biopsy was performed, it shows throbotic microangiopathie. The patient has benifit of plasma exchanges with conventional hemodialysis. The answer of plasma exchange was partial and not sustainable. She benifed later of the ECULIZUMAB, our patient got back a renal function (35 ml/Mn), with normal rate of platelet and hemoglobulin. The genetic study finds that parents as well as her small sister are heterozygous it should be noted that her first cousin dialysis chronic for an indefinite renal disease and benefited from two kidney transplantation.
Results: It is the first case of atypical HUS with MCP deficiency diagnosed in Algeria, the patient answered partially the sessions of plasma exchanges and she reponded well to treatment with ECULIZUMAB with partial recovery of renal function. It is possible that his first cousin is wearing the same affection.
Conclusions: The MCP defenciency is an anomaly of the complement associeted with the atypucal HUS. A therapeutic hope was born with ECULIZUMAB.

P0028

C5a drives the cutaneous lesions in experimental epidermolysis bullosa acquisita, which can be targeted by highly galactosylated IgG1 immune complexes

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Purpose/Objective: Epidermolysis Bullosa Acquisita (EBA) is a chronic autoimmune disease, causing subepidermal blistering of the skin. Autoantibodies targeting type VII collagen are critical for disease development. Previous data suggest that immune complex formation in the skin leads to activation and deposition of complement. Further C5-deficient mice were protected in experimental EBA indicating that complement fragments downstream of C5 may be important for EBA development. Of note, IgG IC can differ in their potential to activate complement and to promote inflammation. In line with this view, we recently uncovered a novel anti-inflammatory pathway by which highly galactosylated IgG1 IC can block C5a-mediated effector functions by an FcgRIIB and Dectin-1-dependent signalling pathway.
Materials and methods: EBA was induced in C57Bl/6J wt, $C5aR^{-/-}$ and Dectin-$1^{-/-}$ mice by repeated transfer of purified rabbit IgG specific to murine collagen type VII. The clinical phenotype was determined by assessment of the affected body surface area. Skin C3b and IgG deposition was determined by immunohistochemistry. To block C5a effects, high and low galactoslyated OVA/anti-OVA IgG1 IC (HiGal-IC and LoGal-IC) were administered prior to the injection of pathogenic typy II collagen antibodies.
Results: We found that $C5aR^{-/-}$ mice are protected from the development of cutaneous lesions. Further, treatment with HiGal- but not with LoGal-IC prior to disease initiation reduced number and size of cutaneous lesions, whereas deposition of collagen type VII-specific antibodies and of C3b was not affected in the two treatment groups. Importantly, HiGal-IC had no protective effect in Dectin-$1^{-/-}$ mice.
Conclusions: C5a promotes the inflammatory responses underlying the inflammatory skin lesions in experimental EBA. The C5a effector functions can be reduced by treatment with HiGal-IC. The galactosylation level of the IgG1 ICs is critical for the inhibitory effects as LoGal-IC are not protective. The missing effect of HiGal-IC in Dectin-$1^{-/-}$ mice suggests an important role for Dectin-1 in in the control of C5a-mediated regulation of autoimmunity.

P0029

C5a receptor signalling in dendritic cells controls the development of maladaptive Th2 and Th17 immunity in allergic asthma

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Purpose/Objective: The mechanism underlying dendritic cell (DC)-mediated induction of maladaptive Th2 and Th17 responses to inhaled allergens in the lung are incompletely understood. Mice lacking C5 or the C5a receptor (C5aR) suffer from maladaptive Th2 immunity in models of experimental asthma suggesting that C5a exerts immunoregulatory properties at the DC/T cell interface. To directly assess the role of C5aR signalling in DCs, we assessed the impact of ovalbumin (OVA) stimulation on DC maturation *in vitro* and DC-mediated experimental allergic asthma induction *in vivo*.
Materials and methods: We characterized DC differentiation from wildtype (wt) and C5aR-deficient BM precursors using GM-CSF by flow cytometric staining. To assess antigen uptake and processing, we incubated DCs with fluorescently labelled OVA. T cell activation and survival were tested in *in vitro* cocultures of wt or C5aR-deficient DCs and OVA-responsive DO11.10 $Rag2^{-/-}$ T cells. To assess the role of C5aR on DC-mediated development of maladaptive immunity in asthma, we adoptively transferred naïve or OVA pulsed wt or C5aR-deficient BM-derived DCs into wt BALB/c mice. Three days before analysis, mice were challenged by intratracheal administration of OVA.
Results: *In vitro*, antigen uptake and processing as well as CD11b expression were reduced in C5aR-deficient DCs. Further IL-1$, IL-6 and IL-23 production were impaired resulting in reduced Th17 differentiation, associated with accelerated T cells death. Additionally we found an increased frequency of $CD11b^{hi}CD11c^{int}Gr\text{-}1^{+}$ $F4/80^{+}$ cells that shared functional characteristics like arginase 1 and nitric oxide synthase 2 expression with myeloid-derived suppressor cells (SC). *In vivo* administration of pulsed wt BM-DCs induced a clear asthmatic phenotype characterized by airway hyperresponsiveness, eosinophilic and neutrophilic infiltration, mucus production and secretion of Th2/Th17 cytokines. In contrast, transfer of C5aR-deficient DCs failed to promote a strong asthmatic phenotype. Further, combined transfer of wt DCs and C5aR-deficient SCs reduced airway inflammation and Th2 cytokine production.
Conclusions: We uncover novel roles of C5aR in Th17 differentiation, T cell survival and differentiation of a SC population controlling Th2 immunity in experimental allergic asthma.

P0030

Comparison of fully activated complement and C5a on the response of retinal pigment epithelial cells

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Purpose/Objective: Retinal pigment epithelial (RPE)-cells undergo cell death during age-related macular degeneration (AMD). This possibly blinding eye disease is related to an enhanced activation of the complement cascade but the influence of the important anaphylatoxin complement 5a (C5a) is unclear. Therefore, in this study complement treated RPE cells were compared to RPE cells treated with isolated C5a.
Materials and methods: Human ARPE-19 cells as a model for RPE cells were treated with increasing concentrations of human complement serum (HCS) or with recombinant C5a. In some experiments zymosan was added to stimulate complement activation. Terminal complement complex C5b-9 was stained by immunocytochemistry. Reactive oxygen species (ROS) were measured by a nitroblue tetrazolium assay. The production of vascular endothelial growth factor (VEGF) in the cell culture supernatants was quantified by ELISA. Polarized RPE cells were grown in transwell inserts.
Results: After 24 h even low amounts of HCS induced a strong C5b-9 specific staining, generated an increase in ROS and clearly raised the VEGF production by ARPE-19 cells. The strongest effects were observed with combined HCS and zymosan treatment. In contrast, C5a treated ARPE-19 showed no C5b-9 staining. There were only slight augmentations in ROS and in the secreted amount of VEGF. Elevated concentrations of C5a and prolonged time of incubation failed to provoke elevated effects. Independent of HCS and C5a treatment, VEGF was mainly secreted towards the basal side.
Conclusions: The influence of C5a induced only minor changes in confluent ARPE-19 cells. In contrast, HCS showed clear effects. These RPE cells may either be relatively resistant against C5a or the effect of C5a may be more transient. In AMD pathology a second trigger might be necessary. For future therapeutic intervention in AMD the inhibition of the complement complement cascade might be more effective than C5a inhibition.

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P0031

Influence of mannose-binding lectin (MBL) deficiency on the clinical presentation of patients with antibody deficiencies

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Purpose/Objective: MBL deficiency, characterized by low MBL plasma levels and/or low-producing *MBL2* genotypes, has only a minor influence on the morbidity of otherwise healthy people, but it becomes symptomatic if other defense defects are present. In this study, we investigated the role of MBL deficiency in the clinical presentation of patients with antibody deficiencies.
Materials and methods: 65 patients with antibody deficiencies were enrolled, including 19 patients with common variable immunodeficiency (CVID), 23 with IgA deficiency (IgAD), 19 with selective IgG4 deficiency (sIgG4D) and 4 with transient hypogammaglobulinemia of infancy. The detection of *MBL2* alterations (-221: X/Y, rs7096206; cod52: A/D rs5030737; cod54: A/B, rs1800450; cod57: A/C, rs1800451) was performed by PCR-RFLP and direct sequencing. Allele and genotype frequencies of *MBL2* compared with those observed in 153 healthy controls.
Results: We did not observe any significant difference on the prevalence of MBL deficiency between patients and controls, except of a higher frequency of the X allele (rs7096206) in patients with IgAD. *MBL2* deficient genotypes were significantly correlated with severe respiratory complications, especially bronchiectasis, in CVID, but did not affect the clinical presentation of IgAD and sIgG4D.
Conclusions: Our findings demonstrate that *MBL2* deficient genotypes might potentiate the clinical symptoms of patients with CVID and are mainly correlated with severe respiratory complications, especially bronchiectasis formation.

P0032

Investigation of the factors that influence complement-dependent cytotoxicity (CDC) following ofatumumab treatment in chronic lymphocytic leukaemia

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Purpose/Objective: CLL patients exhibiting progressive disease often become refractory to standard first line therapies, underpinning the need for novel drugs. An emerging field is targeted monoclonal antibody (MAB) development. In this study we evaluated the ability of the next generation anti-CD20 MAB ofatumumab (OFA), against current first-line anti-CD20 MAB rituximab (RIT), to induce CDC and determined whether complement defects are an issue in CLL MAB treatment regimes.
Materials and methods: CLL cells and serum were taken from patients with confirmed B-CLL diagnosis. Flow cytometric analysis was performed for CDC levels (PI staining) and surface marker expression. Complement activity and levels were verified using CH100 assay, immunonephelometry and ELISA-based assays.
Results: OFA induced significantly higher CDC in CLL primary cells than RIT, with levels of CDC positively correlated with expression levels of CD20. CLL serum sample analysis revealed that >40% of patients were either deficient, or had reduced expression of, one or more complement components, which impacted on their ability to elicit a CDC response to OFA-bound CLL cell line, HG3. Patients with complement levels bordering on normal initially induced a high level of CDC, however on secondary challenge CDC activity in sera was significantly reduced, compared with normal human serum (NHS; $P = <0.0001$; $n = 24$). Importantly, supplementing CLL serum with individual complement components was sufficient to restore CDC activity and protect against exhaustion.C2 deficiency was particularly important, with re-addition of C2 restoring CLL patient serum activity back to NHS levels, ($P = <0.0001$; $n = 11$). Furthermore, high CLL cell burden also contributed to more rapid complement exhaustion.
Conclusions: Our studies demonstrate that OFA is more effective at inducing CDC than RIT and correlates with intensity of CD20 expression on CLL cells. In addition, complement deficiencies in CLL serum suggest that supplementing MAB treatment with fresh frozen plasma, or more specifically C2, may help maintain normal CDC levels, particularly in patients with a high white blood cell count. This study has important implications for CLL patients receiving MAB therapy.

P0035

Polymorphisms causing deficiency in lectin pathway genes: frequency in the Icelandic population

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Purpose/Objective: The complement system has a pivotal role in innate immunity. The lectin complement activation pathway is initiated by either mannan-binding lectin (MBL), or one of three ficolins through associated serine proteases (MASPs). MASP-2 has a central role in initiating the lectin pathway. It is known that homozygotes with the *MASP2* gene mutation *p.D120G* have no functional MASP-2. It is estimated that 1:1000 healthy Danish individuals are homozygous for this mutation *(G/G)*. We evaluated the frequency of *p.D120G* mutation in healthy Icelandic blood donors.
Materials and methods: A total of 453 blood donor samples were genotyped for the mutation *p.D120G* in the *MASP2* gene. Genomic DNA was extracted from EDTA treated blood using salting out procedure and genotyped using 'sequence-specific primer' PCR (PCR-SSP) method.
Results: A total of 37 blood donor samples were found to be heterozygous for this mutation or 8.2%.
Conclusions: Our results show that the frequency of the *p.D120G* mutation is 0.041 in Icelandic population, which is comparable with Danish population (0.039). Next step is to screen for *p.D120G* in variable disease associated populations in Iceland, including individuals with repeated unexplained infections.

P0036

Role of complement regulatory protein CD46 in rheumatoid arthritis

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Purpose/Objective: Rheumatoid Arthritis (RA) is a chronic inflammatory disease characterized by pain, swelling and progressive destruction of multiple joints, affecting approximately 1% of the adult human population. The growing importance of Membrane cofactor protein (MCP; CD46) not only as central membrane bound complement inhibitor but also as critical regulator of T-cell immunity, suggests that regulation of its expression may have significant effects on health and disease. Studies on animal models suggest disease modulating activity of CD 46 in autoimmune disorders. We conducted a case-control study to explore the role of CD 46 in human RA. Till now, only few reports on the expression of CD 46 on circulating cells in RA are present.
Materials and methods: In this study, the surface CD 46 expression on lymphocytes, monocytes and neutrophils of 40 healthy controls and 30 DMARD naïve RA patients were monitored using Flow cytometer. The Circulating Immune Complex (CIC) levels in serum of controls and patients were determined spectrophotometrically. Disease activity scores (DAS28) were monitored in RA patients using swollen and tender joint counts (SJC & TJC), the patient global assessment and the ESR. The correlations of CD46 expression with clinical parameters (DAS28, SJC and CIC) were calculated using Spearman's rank correlation test.
Results: The mean fluorescence intensity values of CD 46 on leukocyte sub-populations were in the order of monocytes >neutrophils > lymphocytes both in controls and patients. In patients, the surface CD 46 expression reduced significantly on lymphocytes followed by monocytes as compared to controls. The mean value of CD 46 expression on neutrophils was comparable between the patients and controls. Significant negative correlations of the monocyte and lymphocyte cell surface CD 46 with DAS28 and SJC were observed in patients. Only lymphocyte CD 46 correlated negatively with CIC.
Conclusions: Decline in the levels of CD 46 expression differentially on different leukocyte subpopulations in patients may contribute not only to the aggravation of complement mediated tissue injury but also to immune deregulation in rheumatoid arthritis. The negative correlations of CD 46 with disease activity indices suggest significant association of CD 46 with the disease state. The role of CD 46 as disease activity marker is envisaged.

P0038

Specificities for microorganisms by Ficolins and Mannan-binding lectin – complement-activating soluble pattern recognition molecules

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Purpose/Objective: We wish to expand the knowledge of soluble pattern recognition molecules (sPRMs) initiating the lectin pathway of complement. This pathway is initiated by the binding of the proteins: mannan-binding lectin (MBL) or one of the three ficolins, H-ficolin, M-ficolin or L-ficolin. These proteins bind to pathogen-associated molecular patterns (PAMPs) displayed by microorganisms. The binding of the sPRMs promotes the activation of associated proenzymes, termed MBL-associated serine proteases, (MASPs). The MASPs then mediate the down-stream activation of complement.
Materials and methods: We have screened a large number of microorganisms (bacteria, viruses and fungi) for binding in serum by H-ficolin, L-ficolin, M-ficolin and Mannan-binding lectin. Quantification of ficolins and MBL was by time-resolved immuno-fluorometric assays (TRIFMA) in serum samples exposed to various microorganisms.
Results: We find a huge difference in binding to different microorganisms by these four sPRMs and a remarkably specificity for certain serotypes of pneumococci and fungi. As another example we find that M-ficolin binds to encapsulated but not to unencapsulated *Streptococcus Agalactiea*, while the reverse was true for MBL.
Conclusions: A highly specified biological role of MBL and ficolins in activation the innate immune system when different microorganisms is exposed to the body is indicated.

P0039

Transcriptomic 'portraits' of transient inflammatory arthritis in C5 complement component deficient mice

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Purpose/Objective: C5 complement component is a central molecule that is essential for leukocytes activation in inflammation. C5 encoding gene is located within the Pgis2 chromosome 2 interval linked to murine proteoglycan-induced spondylitis (PGIS). The goal of the study was searching for genes that rescue the inflammatory phenotype in C5-deficient BALB/c.DBA/2-Pgis2 congenic mice.

Materials and methods: Collagen antibody-induced arthritis (CAIA) was induced in BALB/c, DBA/2 and congenic mice. Mice were sacrificed at the acute inflammation, and collected synovial joints were subject for histopathological analysis. Total RNA was isolated from arthritic paws and analyzed for genechip expression. Serum C5a levels were measured using ELISA. Ingenuity Pathways Analysis and hierarchical clustering of expression patterns were performed.

Results: DBA/2 mice carry natural C5 deficiency and were resistant to CAIA, while C5-suffcient BALB/c were highly susceptible for CAIA. C5-deficient Pgis2-congenic mice demonstrated an intermediate phenotype of transient inflammatory arthritis. Statistical analysis of differential gene expression in the congenic arthritic paws revealed $n = 27$ genes that expression were significantly deregulated ($P < 0.01$) and greater than ±1.5-fold change when compared to arthritic paws of wild-type BALB/c mice.Empirical false discovery rate (FDR) tested for these comparison criteria using 500 permutations was 0% for median FDR and 18.5% for 90-th percentile FDR. Most significantly down-regulated genes include FK506 binding protein 5 (-2.58-fold, $P < 0.0003$), raftlin lipid raft linker 1 (-2.38-fold, $P < 0.008$), serine peptidase inhibitor clade A isoform 3C (-2.18-fold, $P < 0.0008$). Top upregulated genes in Pgis2 mice with attenuated CAIA were sphingosine kinase 2 (2.8-fold, $P < 0.00001$) and defensin beta 3 (2.92-fold, $P < 0.007$). According to IPA, differentially expressed genes showed the enrichment with messages that are specific for macrophages and effector T cells.

Conclusions: The early, transient arthritis in Pgis2-congenic C5-deficient mice is due to multifactorial by committee gene expression and may lead to the identification of novel targets for therapeutic intervention in inflammatory arthritis models.

Poster Session: Granulocytes; Basophils, Mast Cells Oesinophils

P0040
Activities of histamine H2- and H4-receptors on granulocytes

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Purpose/Objective: Eosinophils and neutrophils are involved in the two major lung diseases, allergic asthma and chronic obstructive pulmonary disease (COPD), respectively. The biogenic amine histamine plays a role in allergic diseases as well as in inflammatory diseases, such as COPD. Histamine is recognized on target cells by four different receptors, histamine H_1-receptor (H_1R), H_2R, H_3R, and H_4R. Of these, H_1R, H_2R, and H_4R are expressed on eosinophils, while neutrophils express only H_1R and H_2R. In this study, our objectives were to functionally examine the H_XR subtypes on human eosinophils and to compare the effects of histamine on eosinophils and neutrophils with special emphasis on the regulatory H_2R.
Materials and methods: Granulocytes were isolated from peripheral human blood by density gradient centrifugation followed by hypotonic lysis of erythrocytes. The obtained cell suspension contained at least 95% viable neutrophils. Eosinophils were enriched from the granulocyte suspension by CD16-directed magnetic bead-assisted cell sorting, resulting in preparations consisting of 98% eosinohils. H_XR were targeted by histamine and selective ligands, such as the H_4R-selective agonist UR-PI376, the H_4R-selective antagonist JNJ7777120 and the H_2R-selective ligands amthamine (agonist) and famotidine (antagonist). Intracellular calcium concentrations ($[Ca^{2+}]_i$) were measured by the Fura-2 method and *in vitro* chemotaxis was quantified in a Boyden chamber. Finally, reactive oxygen species (ROS) production was analyzed by reduction of cytochrome c.
Results: Histamine and UR-PI376 induced $[Ca^{2+}]_i$ mobilization and chemotaxis of eosinophils, which were inhibited by JNJ7777120. In contrast, blockade of the H_2R enhanced histamine-induced chemotaxis. Formation of ROS was induced in eosinophils and neutrophils by the formyl peptide fMLP, but not by histamine. In both granulocytes subsets, the induction of ROS formation was reduced by histamine in a H_2R antagonist-sensitive manner.
Conclusions: On eosinophils, histamine has both activating and reducing effects on differing cellular functions. In contrast, on neutrophils histamine via the H_2R mainly reduces activation, probably due to the absence of H_4R expression.

P0041
An evaluation of neutrophil function: a new approach to the chemiluminescent analysis

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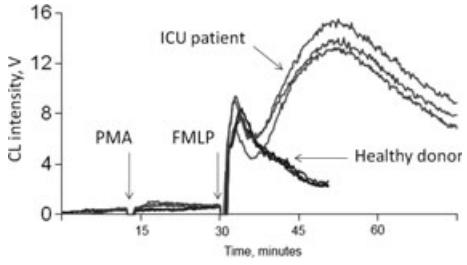
Purpose/Objective: Neutrophils as a part of nonspecific immunity factors play a crucial role in antimicrobial resistance. Reactive oxygen species (ROS) are important compound of the neutrophils' microbicidal action. Analysis of neutrophils' ROS production could provide valuable data on a phagocyte link of immunity. Chemiluminescent (CL) assay being highly sensitive allows evaluating oxidative output of the cells in dynamics.

There were published many controlled studies on neutrophil CL in humans with different diseases. However the results often vary between authors because of the lack of standardized method of CL analysis. So we tried to develop a methodology of neutrophils' CL analysis according to principles of the evidence-based medicine.
Materials and methods: 95 healthy donors and 3 ICU patients with second-third degree burns participated in the study.

To dilute blood samples we used Henks Balanced Salt Saline (HBSS) with glucose, pH = 7.4 supplemented with 2 mM HEPES. Luminol (Sigma-Aldrich) was dissolved in double distillated water at 1 mM. N-formyl-methionyl-leucyl-phenylalanine (FMLP, Sigma-Aldrich) and 4-phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) were diluted in DMSO (MP Biomedicals, LLC) to make stock solutions that were dissolved in HBSS on the day of experiment. CL was evaluated by means of a chemiluminometer Lum-12 (Chair of Biophysics, Moscow State University).
Results: We have obtained curves of CL-response of whole blood neutrophils in healthy donors under conditions ofPMA, FMLP and their combination. A statistical analysis has shown optima for their concentrations and for time interval between blood sampling and CL assay. We have also analysed a blood dilution impact to a CL response. We have developed a method on the evaluation of a neutrophil function that is based on a step-by-step stimulation of the cells by PMA and FMLP.

Using this method, we compared the distributions of CL characteristics for the population of 95 healthy donors.

We also held a CL assay ICU patients (Fig.1) and then compared the results with data from clinical observations and laboratory tests.



Conclusions: As a result we suggest reliable and replicable method on the evaluation of a neutrophil function applicable in clinical practice. We argue that to interpret reference means of the adjusted CL characteristics distributions it is important to foresee future tendencies on septic complications.

P0042
Co-operation between innate CCR3-expressing granulocytes and macrophages in controlling early establishment of Brugia malayi infection

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Purpose/Objective: The mouse is a non-permissive host for the human lymphatic filarial parasite, *Brugia malayi*. Experimental infection with third stage infectious larvae (L3) induces a rapid recruitment and / or expansion of eosinophils and macrophages and >95% of larvae are killed by a host granulomatous response within the first 2 weeks of infection. We investigated the early immune events that govern resistance to primary filarial infection.
Materials and methods: Experimental *B. malayi* infections were undertaken by infection with 50 L3 larvae into the peritoneal cavities of interleukin(IL)-4 receptor alpha$^{-/-}$ IL-5$^{-/-}$ mice, Severe Combined ImmunoDeficient (SCID) mice or WT mice all on a BALB/c

background. Administration of anti-C-C chemokine receptor 3 (CCR3) antibody and clodronate encapsulated liposomes 1 day before infection selectively depleted eosinophils and macrophage populations, respectively. Larvae and peritoneal exudate cells were recovered at between^{+}2 and^{+}14 days. More long-term infections (+12 weeks) were used to examine impact of manipulation of the host response on the establishment of patent adult infections.

Results: We report that severely impaired type 2 signalling (IL-4, -5 and -13) in IL-4Ra$^{-/-}$/IL-5$^{-/-}$ mice produces a susceptible phenotype able to host long-term *B. malayi* fecund infections both in the peritoneal cavity and lymphatics accompanied by a complete ablation of eosinophils at the infection site. Resistance is dictated by early immune events as significantly higher parasite burdens can be discerned in IL-4Ra$^{-/-}$/IL-5$^{-/-}$ mice as little as^{+}2d after infection. By tracking larval attrition during the first 2 weeks following temporal ablation of eosinophils or macrophages, we show both cell types are necessary for resistance to early larval establishment. Increased susceptibility is also apparent in SCID mice following anti-CCR3 targeted ablation of eosinophils indicating the mechanism of early larval attrition is independent of adaptive immunity.

Conclusions: The presence of both CCR3^{+} granulocytes and macrophages are important for optimum resistance to larval *B. malayi* infection. We speculate that recruited CCR3^{+} eosinophilic granulocytes may mediate larval killing by regulating macrophage effector function.

P0043

Expression of HPV16-E7 oncoprotein in skin exacerbates the contact hypersensitivity response to DNCB

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Purpose/Objective: Cervical cancer is the second most common cancer of women worldwide and is the first cancer described to be entirely induced by a virus, the human papillomavirus. This study is aimed to better understand the immunopathology of pre-cancerous skin lesions associated with HPV infection.

Materials and methods: We use C57BL/6 mice expressing HPV16-E7 oncoprotein as a transgene from a keratin 14 promoter in mouse skin (E7 mice). The inflammatory response to DNCB was investigated after application to ear skin of E7 and control mice.

Results: E7 mouse ears undergo increased swelling relative to control ears within the first 8 h (early) following DNCB exposure, and also exhibit increased swelling at day 5 (late) which resolves by10 days after DNCB exposure. E7 skin has two to three-times more mast cells than control, and there were more degranulated mast cells in the basal layer of the epidermis. Mast cells in E7 mouse skin were found to have a greater capacity to degranulate during a passive cutaneous anaphylaxis response. The early response induced by DNCB was accompanied by substantial mast cell degranulation in E7 mouse ear skin, which was not observed in control ear skin. The increased late response to DNCB in E7 mice was accompanied by elevated levels of IL-1b and IL-19, but not IL-33, in the skin.

Conclusions: E7 transgenic skin shows exaggerated swelling in response to a contact sensitising agent, in association with mast cell degranulation and IL-19 release.We are now investigating a possible link between mast cells and IL-19 in the immunopathology of HPV infected skin, particularly as mast cells have been associated with HPV positive intraepithelial neoplasia in humans, and as IL-19 plays a role in psoriasis lesions where skin thickening similar to that in HPV16-E7 skin is observed.

P0044

Functional responses of basophils to endogenous danger signals

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Purpose/Objective: Cellular injury, necrosis or stress that might occur during inflammatory responses or mechanical injury leads to release of damage associated molecular patterns (DAMPs), intracellular molecules that are normally retained inside the cell. These molecules include ATP, uric acid, S100A proteins and HMGB1, which are interpreted as danger signals by the immune system when present in the extracellular milieu. Several DAMPs have been shown to have differential (usually pro-inflammatory) effects on immune cells, including neutrophils and eosinophils, but whether and how basophils respond to endogenous danger signals is unknown.

Since basophils are important players in allergic immune responses and these often involve cellular damage, we aimed to understand how human and mouse basophils react to different DAMPs and how this might contribute to an inflammatory response.

Materials and methods: We cultured human *ex vivo* isolated or mouse *in vitro* differentiated basophils in the presence or absence of different DAMPs and assessed viability, chemotaxis, surface expression of activation markers CD63 and CD203c, and chemotactic responses.

Results: We found that basophils are capable of responding to a variety of different DAMPs and that different DAMPs affect a variety of basophil functions, including survival, migration and cytokine release.

Conclusions: Our results suggest that endogenous danger signals modulate inflammation also through their action on basophils.

P0045

Hsp70 and Hsc70 dynamics in human neutrophils under heat shock conditions

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Purpose/Objective: Neutrophils are short lived cells characterized by low biosynthetic activity, however they are inducible for synthesis of heat shock proteins 70 kDa (HSP70s) possessing cell protective functions. The aim of this study was to analyze the dynamics of HSP70 content in human neutrophils in response to heat shock (HS), and to investigate factors affecting the dynamics.

Materials and methods: Neutrophils isolated by density gradient from heparinized venous blood of healthy volunteers were stained intracellularly with monoclonal antibodies (mAbs) directed to constitutive Hsc70 (SPA815, Stressgen) or inducible Hsp70 (SPA810, Stressgen) proteins, or with HSP70 antibody not distinguished between Hsc70 and Hsp70 (BRM-22, Sigma). Detection of intracellular HSP70 levels was performed by flow cytometry.

Results: In most cases after short-term heating (43°C, 10 min) we registered an increase and following decrease in 15−30 min after the end of HS of HSP70 levels with all three mAbs (BRM-22, SPA810, SPA815). We did not detect any increase phase by Western blotting. Pre-incubation with cycloheximide, inhibitor of protein synthesis, did not change the dynamics of intracellular HSP70 levels revealed by flow cytometry. This fact indicates that the HSP70 increase phase is not mediated by de novo protein synthesis, but is possibly related to the availability of epitopes of HSP70s for binding with mAbs. The decrease phase of HSP70 level was confirmed by Western blotting normalized with β-actin. This phase can be connected with release of HSP70s into extracellular space phenomenon described mostly for Hsp70. Treatment of neutrophils with glybenclamide, ATP sensitive potassium

channel blocker, resulted in the HSP70 decrease stage reduction detected with all mAbs suggesting that Hsc70 but not only Hsp70 was released from the cells. Treatment of neutrophils with NH_4Cl, lysosomotropic compound, resulted in greater increase of intracellular HSP70 level, possibly, through impairment of vesicle maturation.
Conclusions: Thus, our data suggest that both Hsp70 and Hsc70 are released from neutrophils under the HS conditions through the endolysosomal pathway involving ATP-sensitive mechanism.

P0046

Human neutrophils establish cellular interaction with natural killer cells to enhance their interferon-gamma production

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Purpose/Objective: The role of neutrophils in the orchestration of immune responses has been increasingly recognised by their functional interactions with various immune cells, such as dendritic cells, monocytes as well as lymphocytes. Herein, we aimed at defining whether human neutrophils establish a functional cross-talk with natural killer (NK) cells too. In fact, it has been clearly recognised that NK cell activation is finely tuned by accessory cells which provide them with activating signals such as membrane molecules or soluble factors. Based on these premises, the aims of this study were: (i) to evaluate the ability of human neutrophils to activate NK cells in terms of IFN-γ production; (ii) to characterize the cell-cell interactions that could eventually occur between human neutrophils and NK cells.
Materials and methods: Human neutrophils and autologous NK cells were isolated from buffy-coats of healthy donors. NK cells were then cultured alone or with neutrophils at 1:1 ratio and stimulated in the presence of LPS in combination with IL-15 and IL-18, for 18 h. IFN-γ production was then assessed by ELISA. Cell-cell interactions and cell identity were characterized by confocal microscopy and immunohistochemistry.
Results: NK cells showed an enhanced capacity to secrete IFN-γ when cultured in the presence of neutrophils under our stimulatory conditions. Such an increased IFN-γ production by NK cells was found to be dependent on the contact between the two cell types. By the use of specific neutralizing antibodies, we could then identify the specific membrane molecules involved in the NK cell-neutrophil interactions, specifically the CD11d/CD18 integrin on the NK cell, and the ICAM-3 immunoglobulin family member on neutrophil side. The potential *in vivo* occurrence of a neutrophil/NK cell cross-talk was uncovered in the lesions of patients with psoriasis, Crohn's disease and Sweet's syndrome disease, in which a neutrophil-NK cell colocalization is present.
Conclusions: The present work reports the capacity of human neutrophils to potentiate the IFN-γ release by NK cells *via* contact-dependent mechanisms. These data may have potential implications in the pathogenesis of diseases where a neutrophil/NK cell colocalization is observed.

P0047

Mast cell-specific NFATc1-deficiency drives overexpression of the transcription factor NFATc2 in murine bone marrow-derived mast cells

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Purpose/Objective: To investigate the function of the transcription factor NFATc1 in murine mast cells we generated mast cell-specific NFATc1-deficient mice by crossing Mcpt5Cre mice with $NFATc1^{fl/fl}$ animals.
Materials and methods: We produced bone marrow-derived mast cells from these animals and looked after their ability to express the cytokines IL-6 and IL-9 *in vitro*. For this we used qRT PCR and ELISA. Via Western Blotting we checked the expression of the transcription factors IRF-4 and NFATc2.
Results: According to the expectations based on our previous work, the expression of IL-6 was unaffected in the absence of NFATc1. However, the production of IL-9 was enhanced. Detailed analyses revealed the overexpression of the closely related NFATc2 in NFATc1-deficient mast cells. Concomitantly, the expression of the transcription factor IRF-4 and of IL-1β was also strongly enhanced. Thus, the increased expression of IL-9 in the absence of NFATc1 might be explained by the autocrine effect of IL-1β or by IRF-4-mediated transactivation of the IL-9 promotor. How NFATc1 modulates the expression of NFATc2 and the influence of the latter on the production of IRF-4 and IL-1β is currently unknown.
Conclusions: The model used in this study to examine the specific function of NFATc1 in mast cells is not suitable, since the absence of this transcription factor can be compensated by an overexpression of NFATc2. These results, however, show a possible influence of NFATc1 on the production of NFATc2 as part of an intracellular network modulating the expression of IL-9.

P0048

Mast cells phagocyte Candida albicans via Toll-like 2 receptor without TNF-a and IL-10 production

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Purpose/Objective: The pattern recognition receptors of innate immunity are important in defense against pathogens by recognizing of surface molecules and triggering important signaling pathways. *Candida albicans* (*C. albicans*) is recognized by cells of innate immunity by Toll-like 2 receptors. Mast cells have this receptor on cellular membrane and its immune mechanisms include synthesis and secretion of mediators, antigen presentation, as well as phagocytic and microbicidal activities. So, this study evaluated *in vitro* the TNF-α and IL-10 production and phagocytosis by mast cells challenged with *C. albicans* and the involvement of TLR2 in these mechanisms.
Materials and methods: Murine bone marrow cells (BMMC) wild type ($TLR2^{+/+}$) or TLR2 knockout ($TLR2^{-/-}$) were cultured for 21 days in presence of stem cell factor (SCF) and interleukin-3 (IL-3). Mast cells were challenged with FITC-labeled *C. albicans* by 30 or 60 min and the phagocytosis analyzed by confocal laser scanning microscopy. TNF-α and IL-10 production was measured by ELISA after 24 h of challenge with *C. albicans*.
Results: BMMC $TLR2^{+/+}$ did not produce TNF-α or IL-10 after stimulation or not with *C. albicans*. However, these cells challenged with zymosan produced detectable levels of TNF-α, but not IL-10. After 30 min, 30% of BMMC $TLR2^{+/+}$ showed internalized *C. albicans*, approximately four yeasts per cell. Besides, the BMMC $TLR2^{-/-}$ did not produced TNF-α or IL-10 irrespective of experimental conditions and their phagocytosis was also impaired. After 30 and 60 min, 98.4% and 99.6% of mast cells, respectively, had not internalized fungi. The percentage of zymosan phagocytosis by mast cells was also very low (2.7%).
Conclusions: Data demonstrated the phagocytic capacity of BMMC against *C. albicans* yeasts and the strong involvement of the TLR2 receptor in this mechanism. Still, the absence of TNF-α and IL-10 production after fungal challenge may represent an immune escape of the *C. albicans* after their internalization by mast cells.

P0049
Neutrophil chemotaxis is impaired in celiac disease

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Purpose/Objective: Celiac disease (CD) is triggered by the ingestion of gliadin, the immunogenic component of gluten-containing grains. We have observed that exposure to gliadin induces rapid and massive influx of neutrophils to the murine gut mucosa, suggesting it is a chemoattractant factor for neutrophils. Aims of this study; has gliadin also chemoattractant properties for human neutrophils, and do neutrophils from healthy individuals (HC) and CD patients respond differently to gliadin.
Materials and methods: Neutrophils were isolated from venous blood of HC and CD patients and applied in an under-agarose assay to monitor neutrophil chemotaxis to pepsin-trypsin-digested gliadin (PTG) or N-formyl-methionyl-leucyl-phenylalanine (fMLP) as a positive control. Resting neutrophils and PTG- or fMLP-stimulated neutrophils were analyzed by flow cytometry for CD62L surface expression.
Results: Human neutrophils migrated towards PTG and fMLP. However, compared to the chemotactic response of HC neutrophils to PTG (5.7 ± 1.3 net neutrophil migration), the chemotactic response of CD neutrophils was markedly reduced (0.4 ± 0.3 net neutrophil migration, $P < 0.001$). A similar, albeit non-significant difference was also observed in CD versus HC neutrophil migration to fMLP (4.8 ± 1.1 versus 11.9 ± 2.9 net neutrophil migration, respectively, $P = 0.067$). The percentage of CD62L-expressing neutrophils (resting 67 ± 15) diminished after fMLP- (52 ± 22) and PTG-stimulation (44 ± 19) in HC, but remained unchanged in CD (resting 86 ± 5, fMLP (61 ± 24), PTG (90 ± 6).
Conclusions: These results suggest that PTG is also a chemoattractant factor for human neutrophils. Compared to HC, the CD neutrophil chemotactic response to PTG and fMLP is impaired in the under-agarose assay, suggesting that CD neutrophils have reduced chemotactic potential that possibly involves L-selectin.

Granulocytes; Basophils, Mast Cells Oesinophils

P0050
Redox regulation of degranulation in human neutrophils

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Purpose/Objective: The NADPH oxidase of neutrophilic granulocytes is a complex enzyme consisting of membrane and cytosolic subunits. After activation of the cells the cytosolic subunits translocate to the membrane where the functioning oxidase complex is formed. The active enzyme carries out the transfer of one electron from cytosolic NADPH oxidase to molecular oxygen, generating thereby superoxide anion in the exterior of the cell or in the phagocytic vacuoles and H^+, $NADPH^+$ in the cytosol. It was demonstrated by Jankowski and Grinstein (1999) that NADPH oxidase is able to transfer electrons against a potential gradient. Thus, the activity of NADPH oxidase is not only restricted to destruction of invading microorganisms, but it can rapidly change the membrane potential causing a depolarization.

The degranulation plays a principal role in most proinflammatory functions of neutrophils. However the relationships between a membrane potential and degranulation was not investigated completely. In order to investigate the regulatory role of redox potential on degranulation, we tested the inhibitors of NADPH oxidase, diphenylene iodonium (DPI) and apocynin, in the *in vitro* model of CB/fMLP-activated human neutrophils.
Materials and Methods: Exocytic insertion of CD63 and CD66b into the cell membrane was determined by flow cytometry. Radical oxygen species (ROS) production was measured using luminol chemiluminescence method.
Results: Activation of neutrophils with CB/fMLP resulted in a high release of azurophil and specific granules. Adding the DPI in several concentrations before activation of the cells resulted in inhibition of azurophil and specific granules release, while a specific inhibitor of enzyme complex, apocynin, caused a dose-dependent suppression of degranulation of CB/fMLP-activated cells.
Conclusions: The present study showed that inhibition of NADPH oxidase, responsible for the depolarization of membranes, resulted in downregulation of degranulation of activated cells that can be caused by the deterioration of fusion proteins interaction with the membranes.

P0051
Role of the CREB transcription factor in inflammatory cytokine production by human neutrophils

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Purpose/Objective: Neutrophils influence innate and adaptative immunity by generating numerous mediators whose regulation largely depends on transcription factors typically associated with inflammation, such as NF-kB and C/EBP factors. Here, we show that this functional response also involves the CREB transcription factor.
Materials and methods: Protein analyses by immunoblot and ELISA; gene regulation analyses by EMSA, qPCR, ChIP assays, and luciferase assays. Most studies in primary neutrophils, except those featuring transient overexpressions or luciferase assays, which were carried out in DMSO-differentiated, granulocytic human PLB-985 cells.
Results: Neutrophil stimulation with physiological agonists (LPS, TNF) led to a rapid and transient CREB DNA-binding activity, and concurrent phosphorylation of CREB1 on S133. Accordingly, the same stimuli elicited the transactivation of a CREB-driven luciferase reporter in neutrophil-like PLB-985 cells. Functionally, overexpression of a dominant negative CREB mutant (K-CREB) or of a point mutant (S133A) resulted in a decreased ability of neutrophilic PLB-985 cells to generate inflammatory cytokines (CXCL8, CCL3, CCL4, TNFa). In primary neutrophils, CREB DNA binding and S133 phosphorylation were found to occur downstream of the p38 MAPK/MSK1 axis. The parallel phosphorylation of another bZIP transcription factor, C/EBPb, was similarly affected. Inhibition of p38 MAPK or MSK1 prevented cytokine generation in neutrophils, as well as the recruitment of either P-CREB or P-C/EBPb to chemokine promoters in ChIP assays.
Conclusions: Collectively, our data show the involvement of CREB in neutrophil cytokine production, the key role of its S133 residue, some of the molcular mechanisms involved, some of the upstream signaling events, and the parrallel activation of another bZIP factor. Our study also identifies potential molecular targets that could be exploited in the context of several chronic inflammatory diseases that prominently feature neutrophils and their products.

P0052

Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) negatively regulates the oxidative burst in human phagocytes

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Purpose/Objective: The production of reactive oxygen species (ROS) is an important effector mechanism mediating intracellular killing of microbes by phagocytes. Inappropriate or untimely ROS production can lead to tissue damage, thus tight regulation is essential. We recently characterized Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) as an inhibitory receptor expressed by human phagocytes. We now set out to study its role in the regulation of these cells.
Materials and methods: Primary human monocytes and neutrophils were used to study bacterial killing, oxidative burst and SIRL-1 expression upon activation with various stimuli. SIRL-1 signalling was analyzed in transfectants of wild type and mutated SIRL-1. In a cohort of RSV-infected infants, we studied SIRL-1 expression on neutrophils in BAL and peripheral blood.
Results: We demonstrate that ligation of SIRL-1 dampens Fc receptor-induced ROS production in primary human phagocytes. In accordance, SIRL-1 engagement on these cells impairs microbicidal activity of neutrophils, without affecting phagocytosis. The inhibition of ROS production may result from reduced activation of extracellular signal-regulated kinase (ERK), since co-ligation of Fc receptors and SIRL-1 on phagocytes inhibited phosphorylation of ERK. Importantly, we demonstrate that microbial and inflammatory stimuli cause rapid down-regulation of SIRL-1 expression on the surface of primary neutrophils and monocytes. In accordance, SIRL-1 expression levels on neutrophils in bronchoalveolar lavage fluid from patients with neutrophilic airway inflammation are greatly reduced.
Conclusions: We propose that SIRL-1 on phagocytes sets an activation threshold to prevent inappropriate production of oxygen radicals. Upon infection, SIRL-1 expression is down-regulated, allowing microbial killing and clearance of the pathogen.

P0053

Subpopulations of blood cells forming the mast cells — lymphocytes rosettes

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Purpose/Objective: Lymphoid cells are able to contact with mast cells.
Materials and methods: In the investigation the methods of MLR-forming and CD-determination were used.
Results: Lymphoid cells of peripheral blood in patients with cancer of larynx forms increased number of so-called mast-lymphocytes rosettes (MLR) in comparison with patients with precancer diseases and healthy donors. Herewith the number of $CD5^+$, $CD11^+$, $CD16^+$ and $CD54^+$ blood lymphocytes was decreased in patients ofboth groups plus decreased level of $CD25^+$ cells in larynx cancer patients and the number of CD 20^+ , $CD95^+$ and $CD3^+$ lymphocytes was (had) at the normal level. Analysis of subpopulations of lymphocytes that forms the contacts with mast cells revealed the predominant participation in MLR-forming $CD3^+$ and $CD25^+$ cells in patients with larynx cancer and $CD19^+$ and $CD11^+$ in patients with precancer diseases.
Conclusions: So the subpopulation compositions of lymphoid cells contacting with mast cells are varied in different diseases.

P0054

T4 phages head proteins do not induce production of reactive oxygen species by granulocytes

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Purpose/Objective: Phages are viruses specific to bacteria. Their application in drug-resistant bacterial infections has become significant alternative for antibiotics. However, phage therapy still evokes discussion due to its safety. Studies of phage influence on immunological system are necessary to provide more profound understanding of their safety and effectiveness. In this work we evaluated the effect of T4 head proteins on reactive oxygen species (ROS) production by granulocytes.
Materials and methods: Highly purified, by two-step affinity and size-exclusion chromatography (<1 Eu/ml LPS), recombinant T4 phage head proteins gp23, gp24, gphoc and gpsoc were used with controls: PBS, albumin (alb), LPS, PMA. LPS content was assessed by the Chromogenic End point LAL test. Granulocytes (PMN) were isolated from buffy coats of human healthy volunteer blood using double gradient Histopaque. ROS formation was measured using luminol-dependent chemiluminescence (CL) assay in microplate reader. The reaction mixture (100 μl) for CL in each well contained: 10^6 cells/ml, 10 μg/ml protein preparation or 500 Eu/ml LPS or 1 μM PMA, 10 mM luminol. Measurements were carried out at 37°C for 1.5 h.
Results: Highly purified T4 phage proteins: gp23, gp24, gphoc, gpsoc did not induce ROS production (the same level as negative controls) during the whole period of the test. As expected, PMA and LPS highly increased ROS formation.
Conclusions: PMN are known to respond immediately after a virus infection, e.g. HCV core protein evokes intensive ROS production by blood cells initiating inflammatory process. Excessive ROS production may cause cellular damage and induce inflammatory reactions which may be dangerous to patients.Our results indicating that phage proteins do not induce ROS provide good argumenst for the safety of phage therapy.

P0055

The functional features of neutrophils in sportsmen with different level of training loadings

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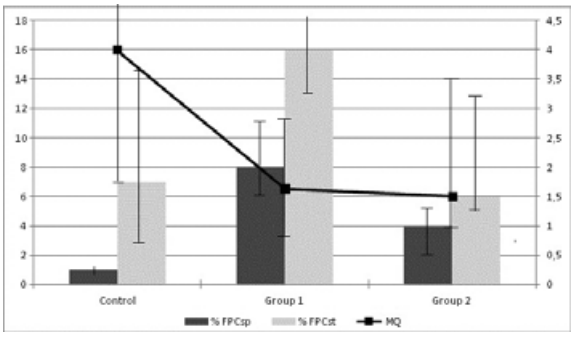
Purpose/Objective: To investigate the influence of training loadings on phagocytic, oxidized activity and the production of cytokine by neutrophils.
Materials and methods: Functional activity of neutrophils was studied in two groups of sportsmen: group 1 ($n = 9$) — highly qualified sportsmen with high level of training loadings (6—12 times a week for 2 h); group 2 ($n = 11$) — sportsmen with low level of training loadings (<3 times a week). The untrained volunteers served as the control ($n = 96$). Research spent to the intercompetitive season of a year training cycle. We defined the quantity of neutrophils, their capacity to ingest zymosan in phagocytosis assay, oxidized activity in spontaneous and zymosan-stimulated NBT-test, and their ability to secrete cytokines IL-1b, IL-6, IL-8, IL-10 and IL-1RA at rest or upon subsequent stimulation with LPS or zymosan. ELISAs were used to measure cytokine levels in neutrophil-derivated supernatant.
Results: Highly qualified sportsmen had a lower relative quantity of blood neutrophil compared with the controls ($P < 0.05$). The neutro-

phil phagocytic capacity was on 31.11% lower ($P < 0.01$) in highly qualified sportsmen and on 24.44% lower ($P < 0.01$) in group 2 compared with the controls. Neutrophils of sportsmen from both groups had considerably raised spontaneous oxidized activity. Quantity of formazan-positive cells (FPC) was raised after stimulation by zymosan too, however mobilisation quotient (ratio %FPC in stimulated NBT-test to %FPC in spontaneous NBT-test) was more than in two times below compared with the controls ($P < 0.05$). Neutrophils of sportsmen from group 2 possessed the raised ability to secrete cytokines as it is spontaneous, and at stimulation. Spontaneous production IL-1b, IL-8 and IL-1RA by neutrophils was above control value in 10.6 ($P < 0.01$), 4.3 ($P < 0.001$) and in 2.7 times ($P < 0.01$), accordingly. They also produced considerable quantity IL-6 which was not defined in neutrophil-derivated supernatant in untrained subject. Quantity of produced cytokines by neutrophils of highly qualified sportsmen did not differ from control (except for secretion IL-1RA by neutrophils of some sportsmen).



Conclusions: The revealed functional features of neutrophils in sportsmen with different level of training loadings and, especially, distinctions in their capacity to produce cytokines, possibly, are reflectance of a various degree of manifestation of adaptive processes, directed on prevention of pathophysiological inflammatory reactions.

P0056

The inflammatory effect of *ORMDL3* and trimellitic anhydride on mast cells

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Purpose/Objective: Allergic rhinitis has a multi-factorial origin, including genetic and environmental factors. *ORMDL3* is a genetic factor associated with asthma and allergic rhinitis. Occupational rhinitis, which develops in the work place, is an environmental factor. The causative agents are divided into low molecular weight (LMW) and high molecular weight (HMW) substances. In this study, we investigated the mechanism by which *ORMDL3* and trimellitic anhydride (TMA), an LMW substance, affected the release of chemical mediators from mast cells.

Materials and methods: We evaluated the β-hexosaminidase release from stable cloned rat basophilic leukemia (RBL-2H3) cell lines overexpressing *ORMDL3*. To examine the effect of LMW substances, we examined LMW substances-induced β-hexosaminidase release. In addition, we incubated the cells with TMA and analyzed the cell lysates and mRNA by immunoblotting and real-time polymerase chain reaction (PCR) to determine whether TMA affected cellular signal transduction.

Results: The overexpression study revealed that *ORMDL3* enhanced degranulation on stimulation by antigens at low concentrations. We found that among LMW substances, only TMA induced β-hexosaminidase release. Further, TMA elicited degranulation in the absence of Ca^{++}, suggesting that this phenomenon was independent of FcεRI-mediated signal transduction. Immunoblotting analysis revealed that TMA completely blocked the phosphorylation of extracellular signal regulated kinases (ERKs). Thus, there was a possibility that TMA affected mast cells through an unknown signaling pathway.

Conclusions: We concluded that overexpression of *ORMDL3* and TMA exposure resulted in increased inflammation. The effect of TMA exposure was lower than FceRI-mediated degranulation. However, persistent chronic inflammation induces asthma. Therefore, occupational rhinitis should be diagnosed as soon as possible.

P0057

The oxidative burst, but not elastase is essential for fungal clearance *in vivo* in a mouse model of Aspergillus fumigatus pneumonia

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Purpose/Objective: The fungus *Aspergillus fumigatus* and its conidia are ubiquitously present in the environment. In immunocompromised individuals, i.e. during tumour treatments and transplantations, inhaled conidia cause invasive aspergillosis (IA) with a mortality rate over 50%. Polymorphonuclear neutrophils (PMN) are the main innate immune effector cells responsible for clearance of such fungi. For this they employ their potent effector mechanisms, i.e. phagocytosis, the oxidative burst and releasing intracellular granules containing antimicrobial proteins like cathepsin G or elastase (ELA) and inflammatory mediators.

Materials and methods: To study the role of PMN and the contribution of oxidative or non-oxidative effector functions in IA, we analysed knockout mice after infection with the *A. fumigatus* wild type strain ATCC 46645. Anesthetized mice were infected intratracheally with 10×10^6 conidia that were sublethal to wild-type (WT) mice. The infected animals were observed over a period of 14 days, and clinical performance and survival were monitored. Some mice were sacrificed 24 h after inoculation, and fungal outgrowth was determined in lung homogenates.

Results: As a positive control, PMN of WT mice were depleted with a Gr-1 specific antibody (clone RB6-8C5) resulting in a sustained neutropenia and a death within 3 days post infection. In $p74^{phox-/-}$ and $gp91^{phox-/-}$ mice, the NADPH oxidase complex is not functional due to a lack of the cytosolic $p47^{phox}$ or membrane $gp91^{phox}$ subunits, respectively. Infected $p47^{phox-/-}$ and $gp91^{phox-/-}$ mice died within the first 3–4 days and had significant higher numbers of fungal colony-forming units in lung homogenates compared to WT mice. These results indicate an essential role of the oxidative burst for fungi clearance *in vivo*. Surprisingly, in neutrophil ELA deficient ELANE mice the survival after infection was unimpaired and comparable to WT mice indicating that ELA is not essential for immunity against IA. Nevertheless, a delayed *A. fumigatus* clearance was detected in the lung homogenates indicating a partial contribution of ELA to fungal immunity.

Conclusions: Taken together, PMN are essential in *A. fumigatus* elimination. The main effector function for fungi clearance require the oxidative burst whereas ELA as a major component of azurophilic granules is not essential for survival after infection with this pathogen.

P0058

The pathogenesis of the anti-phospholipid syndrome: Toll-like receptor mediated signals in participating neutrophils

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Purpose/Objective: The anti-phospholipid syndrome (APS) is a systemic autoimmune disease characterized by an adaptive immune response against self phospholipid (PL)-binding proteins. Patients are suffering from recurrent thrombotic or thrombembolic events and fetal losses. Although APS is considered as an autoantibody-mediated disease, there is growing evidence that anti-phospholipid antibodies (aPL) are necessary but not sufficient for the clinical manifestations of the syndrome. Analyzing the participation of aPL in pregnancy loss during APS revealed that aPL have a direct impact on complement activation. Beyond this, a role for neutrophil activation by the tissue factor/PAR2 axis has been demonstrated to play an additional role. Finally, microbial infections have been reported to act as triggers for the production of autoantibodies cross-reacting with PL-binding proteins as well as inflammatory stimuli that potentiate the aPL thrombogenic effect. Altogether, these findings suggest a role for the innate immunity in APS pathogenesis. Recently, also Toll-like receptor (TLR) mediated signals have been implicated in the activation cascade of aPL induced thrombus formation. There is evidence that aPL may activate endothelial cells and monocytes through TLR-4-dependent signalling. Whether or not TLR in conjunction with aPL may influence the activation status of neutrophils is not known and is the focus of this project.
Materials and methods: To address this question, we purified polymorphonuclear neutrophils (PMN) from healthy donors and analysed the impact of a purified human monoclonal aPL in the presence or absence of a TLR agonist on neutrophil effector functions.
Results: We found that aPL alone were only able to induce minor activation of PMN effector functions. However, in the additional presence of LPS the activation threshold was markedly lowered shown by an elevated production of ROS, increased phagocytic activity and L-selectin shedding as well as enhanced IL-8 production.
Conclusions: These results suggest that PMN as important innate immune effector cells are directly activated by aPL under inflammatory conditions and therefore may be important contributors to the pathophysiology of APS.

P0059

The role of the small GTPase RhoH in eosinophil development and eosinophilic disorders

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Purpose/Objective: The small atypical GTPase RhoH is highly expressed in eosinophils and further upregulated in patients with hypereosinophilic syndrome (HES), a condition characterised by chronically elevated eosinophil levels and tissue damage with sometimes fatal outcome. The function and regulation of RhoH in eosinophils is unknown. We therefore wished to determine the role of RhoH in eosinophil function and development under normal and pathological conditions.
Materials and methods: Eosinophils were isolated from peripheral blood of healthy donors or HES patients. Expression of RhoH in eosinophils with or without IL-5 stimulation was analysed by Western blot. Transcription factors expression was analysed in an eosinophilic HL60 subline stably transduced with inducible RhoH. Human bone marrow cells were differentiated into eosinophils and RhoH expression was analysed by Western blot. Human or mouse bone marrow from $RhoH^{-/-}$ or wild type mice was differentiated *in vitro* into eosinophils. Cells were analysed by light microscopy or flow cytometry.
Results: We find RhoH is upregulated in some HES patients and that IL-5 upregulates RhoH in eosinophils from normal donors but not patients. Furthermore, it is upregulated during *in vitro* differentiation of eosinophils. $RhoH^{-/-}$ mice have elevated eosinophils levels in blood and bone marrow and *ex vivo* bone marrow eosinophils appear to be more mature, with altered expression of several functionally important surface receptors. *In vitro* differentiation into eosinophils with IL-5 is also enhanced in $RhoH^{-/-}$ bone marrow cultures. Enforced expression of RhoH alters the expression of transcription factors involved in eosinophil development.
Conclusions: The results suggest a regulatory role of RhoH in eosinophil development and function, possibly via negatively regulating IL-5 signalling and/or by affecting the balance of transcription factors regulating eosinophil development.

Poster Session: Immune Evasion

P0060

A murine air pouch model to study group A streptococcal pathogenesis and protective immunity

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Purpose/Objective: Group A Streptococcus (GAS) is a major human pathogen worldwide, responsible for both local and systemic infections. In this work we apply a novel murine model to study GAS infection, in particular the role of a well-known virulence factor the chemokine-degrading protease SpyCEP. Moreover, we extended the application of the air pouch model to evaluate protective immunity.
Materials and methods: Dorsolateral air pouches were inflated in CD1 mice by subcutaneous injection of 3 ml air on day 1 and day 4. On day 6, 1×10^7 CFU of exponential phase 3348 wild type or 3348Δ*spyCEP* knock out strains were injected into the pouch. At 2, 4 or 24 h after infection, the animals were euthanized, and an air pouch lavage was performed. Lavage samples were processed for the parallel analysis of bacteria, cell recruitment and chemokines. Bacterial load was determined by viable counts. Cells were stained with a combination of different cell markers and analyzed by flow cytometry. Chemokine concentrations were measured by a bead-based immunoassay. For protective immunity experiments mice were immunized with adjuvant only, M protein, or a combination of GAS antigens (including SpyCEP) recently shown to be protective in traditional models. After air pouch inflation mice were infected with 3348 strain and bacterial viable counts were performed after 24 h.
Results: To investigate SpyCEP action *in vivo*, we adapted a mouse air pouch model of infection for parallel quantification of bacterial growth, host immune cell recruitment and chemokine levels *in situ*. Mice infected with 3348Δ*spyCEP* showed a tendency to have more neutrophils and monocytes compared to mice infected with the wild type 3348 strain. Concomitantly, the chemokines KC, LIX, and MIP-2 were drastically increased in mice infected with the SpyCEP knockout strain, and growth of this mutant strain was reduced compared to the wild type. We also adapted the model to evaluate protective immunity, and observed that vaccinated groups showed significant bacterial growth reduction compared to the group immunized with adjuvant only.
Conclusions: Taken together, our data suggest that the air pouch model promises broad application in the study of immune system response after streptococcal infection.

P0061

Candida albicans evades protective immunity by promoting the expansion of induced $CD4^+$ $CD25^+$ $FoxP3^+$ Helioslo regulatory T cells that enhance Th17 responses

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Purpose/Objective: Regulatory T cells (Treg) play a central role in limiting helper T cell (Th) responses and maintaining immune homeostasis during infection through their ability to inhibit immune responses. Two distinct subsets of $FoxP3^+$ Treg have been identified; natural Treg (nTreg) are generated in the thymus and appear to be important in self tolerance, whilst induced Treg (iTreg) differentiate from naïve T cells in the periphery in response to invading microorganisms in an antigen-specific manner. *Candida albicans* is found harmlessly on up to 80% of humans, however, superficial infections can become debilitating whilst more serious systemic infections are a common problem within intensive care units. The fungus induces the expansion of both Th1 and Th17 that limit, but do not clear infection. The aim of this work was to investigate the role of Treg cells that may prevent sterilising immunity during *C. albicans* infection.
Materials and methods: C57BL/6 mice or hCD2FoxP3 reporter mice were infected with *C. albicans* for 7 days. Splenic Th cells were isolated and stimulated with C. albicans antigens for a further 5 days. Proliferation and cytokine production were measured by 3H-thymidine incorporation, cellular ELISA and flow cytometery.
Results: We demonstrate for the first time, in a murine model of disseminated *C. albicans* infection, that both yeast and hyphae induce MHC class II dependent Th differentiation towards a Treg phenotype and that these Treg inhibit *C. albicans*-induced Th1 and Th2 cells, but enhance Th17 responses. The expanded Treg cells are largely derived from a FoxP3 negative population, confirming that they are not Treg in origin. Furthermore, these cells are negative for Helios, a transcription factor reportedly expressed in nTreg but not iTreg. Interestingly, *C. albicans* induced $FoxP3^+$ cells co-express ROR-γt suggesting that they may contribute to both Treg and Th17 responses. Importantly, mice depleted of $FoxP3^+$ Treg *in vivo* showed reduced kidney burdens compared to mice with normal $FoxP3^+$ Treg numbers.
Conclusions: These results show that *C. albicans* induces the expansion of iTreg cells that may prevent an effective immune response. We hypothesise that *C. albicans* can escape immune clearance by inducing the conversion of Th17 to Treg cells that limit protective Th1 responses.

P0062

Dynamic induction of immune escape by activated T cells via regulation of B7-ligands on myeloid leukemia cells

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Purpose/Objective: The costimulatory molecules belonging to B7 ligand superfamily can be expressed by acute myeloid leukemia (AML) cells. The significance of B7 ligands in disease progression has been demonstrated by independent clinical studies. Interestingly, presence of the most potent costimulatory molecules B7-2 (CD86) and B7-H2 (ICOS-L) on AML cells has been associated with bad prognosis and disease severity. Here, we aimed to modulate the expression of B7 molecules on myeloid leukemia cells and used them as a model to study the interaction between helper T cells and leukemic blasts.
Materials and methods: The model was established with HL-60 AML cell line and iHL-60 cells generated after protein kinase C (PKC) induction. Flow cytometric analyses, cell sorting, and CFSE-based proliferation assays were used for the determinastion on T cell and/or myeloid leukemia cells immunophenotype and proliferation. ELISA arrays were also performed for the determination of proinflammatory cytokine production.
Results: PKC induction increased B7-2 on HL-60 cell line (designated as iHL-60). The expression of other critical costimulatory molecules such as CD70, TRAIL, CD58, and OX40L expression was not significantly modulated. In the ELISA arrays of human proinflammatory cytokines, only IL-8 differed between HL-60 and iHL-60. The majority (>80%) of HL-60 and iHL60 cells was B7-1^-, B7-$H1^-$, B7-$H2^+$, B7-$H3^+$, and B7-$H4^-$. B7-2 and B7-DC expression was upregulated upon PKC induction. $CD4^+$ T cell responses (CD154,

CD25, CD69 expression and proliferation) were supported by HL-60 and to a higher extend by iHL-60. The presence of B7-2 was not critical for immune stimulation. Intriguingly, especially iHL60 cells expressed B7-H1 (PD-L1), increased B7-DC (PD-L2), and decreased B7-H2 levels soon after the engagement with activated T cells. In the co-cultures, T cells expressed PD-1. In the presence of activated T cell-conditioned iHL-60, T cell activation and proliferation were significantly hampered. Addition of an ICOS-stimulating mAb did not enhance T cell proliferation whereas blockage with PD-1-Fc did. In addition, the expression of CD25 and CD127 on $CD4^+$ T cells was also significantly modulated with different co-culture partners.
Conclusions: In conclusion, immune escape of $B7\text{-}2^+$ and $B7\text{-}H2^+$ myeloid leukemia cells can be triggered by helper T cell responses and potential targeting of PD-1 ligands may be considered for leukemia immunotherapy.

P0063

Evasion of human innate immunity without antagonizing TLR_4 by mutant Salmonella enterica serovar Typhimurium having penta-acylated lipid A

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Purpose/Objective: Bacterial lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, is a good target for mammalian cells to recognize bacterial invasion and trigger innate immune responses. Lipid component of LPS called lipid A plays a critical role in interaction with a mammalian TLR4/MD-2 receptor complex. A hexa-acylated type of lipid A which is relatively conserved among a wide variety of Gram-negative bacteria is strongly recognized by mammalian cells and elicit robust immunological activity but less-acylated types of lipid A isolated from some bacterial species have been shown to have weaker stimulatory activity. Modification of a lipid A to a less acylated form is therefore suggested to facilitate bacterial evasion of host innate immunity. In this study, we have investigated the contribution of less-acylated lipid A to interactions of whole bacterial cells with host cells (especially in humans).
Materials and methods: Mutant strains of *Salmonella enterica* serovar Typhimurium having fewer Lipid A acyl groups were generated by deletion of genes for acyltransferases participating in the biosynthesis of lipid A. LPS, formalin-killed bacteria and live bacteria preparations of these strains were used to stimulate human U937 macrophages. Productions of pro-inflammatory cytokines in the culture supernatants were assayed by ELISA.
Results: The major lipid A type in wild-type (WT) and the mutant KCS237 strain is hexa-acylated; in mutant strains KCS311 and KCS324 it is penta-acylated; and in KCS369 it is tetra-acylated. WT and KCS237 formalin-killed and live bacteria, as well as their LPS, strongly stimulated production of pro-inflammatory cytokines in human U937 cells; this stimulation was suppressed by TLR4 suppressors. LPS of other mutants produced no agonistic activity, but strong antagonistic activity, while their formalin-killed and live bacteria preparations had weak agonistic and no antagonistic activity. Moreover, these less-acylated mutants had increased resistance to phagocytosis by U937 cells.
Conclusions: Our results indicate that a decrease of one acyl group (from 6 to 5) is enough to allow *Salmonella* to evade human innate immunity and that the antagonistic activity of less-acylated lipid A is not utilized for this evasion.

P0064

Investigating the impact EBVs immune strategies have on antigen presentation and $CD8^+$ T cell recognition during lytic cycle replication

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Purpose/Objective: During lytic cycle replication Epstein-Barr virus (EBV) expresses at least three immune evasion genes, BNLF2a, BGLF5 and BILF1. In this study we aim to assess the impactthese genes have on antigen presentation and cytotoxic T-lymphocyte (CTLs) recognition of EBV infected cells.
Materials and methods: Lytically replicating EBV infected cells, in which the expression of these genes is silenced, were used to probe recognition by CTLs, of antigens expressed at different phases of lytic cycle replication.
Results: BNLF2a deficient cells were more efficiently recognised by CTLs specific for immediate early and early expressed antigens, relative to those lacking BGLF5 and BILF1. Whereas, BILF1 deficient cells were more efficiently recognised by CTLs specific for late antigens, relative to BNLF2a and BGLF5 lacking cells.
Conclusions: This suggests BNLF2a plays a dominant role in interfering with antigen presentation at IE and E stages of lytic cycle, while BILF1 plays the dominant role at L stages.

P0065

Role of CD163 in the mycobacterium leprae survival and persistence in lepromatous macrophages

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Purpose/Objective: Lepromatous (LL) macrophages possess a regulatory phenotype that contributes to the immunosuppression observed in leprosy. CD163, a scavenger receptor that recognizes hemoglobin-haptoglobin complexes, is expressed at higher levels in lepromatous cells although its functional role in leprosy is not yet well-established.
Materials and methods: Skin biopsies from LL ($n = 6$) or tuberculoid ($n = 6$) patients were used for immunohistochemistry, immunofluorescence and Western blotting analysis. Sera from LL ($n = 5$), tuberculoid ($n = 5$) and healthy volunteers were used to assess sCD163 levels. Human monocytes were isolated from PBMCs obtained from healthy donors ($n = 6$) and infected with *M. leprae* (ML, MOI 5:1). Cytokine concentrations in cell supernatants were evaluated by ELISA. IDO, CD209 and CD163 expression were evaluated by flow cytometry. Alternatively, HEK293 transfected with cDNA for CD163 was used.
Results: We herein demonstrate that LL lesions are microenvironments rich in CD163 − IDO positive cells. Isolated cells from these lesions are $CD68^+$ IDO^+CD163^+ while higher levels of sCD163 in lepromatous sera correlate positively with IL-10 levels and IDO activity. Different ML concentrations in healthy monocytes likewise revealed a positive correlation between increased concentrations of the mycobacteria and IDO, CD209, and CD163 expressions. The regulatory phenotype in ML stimulated monocytes is accompanied by increased TNF, IL-10, and TGF-β levels whereas the IL-10 blockade reduces ML − induced CD163 expression. The CD163 blockade also reduces ML uptake in human monocytes. ML uptake was higher in HEK293 cells transfected with the cDNA for CD163 than in non transfected cells. At the same time, increased CD163 expression in lepromatous cells seems to be dependent on ML uptake besides contributing to augmented iron storage levels in lepromatous macrophages.

Conclusions: Altogether, these results suggest that CD163 expression induced by ML modulates the host cell phenotype to create a favorable environment for mycobacterial entry and survival.

P0066

The transcription factor Nrf2 inhibits autoimmune neuroinflammation via heme oxygnease-1 expression in dendritic cells

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Purpose/Objective: The transcription factor NF-E2-related factor 2 (Nrf2) orchestrates a protective response against autoimmune neuroinflammation, via a mechanism that remains to be established. We hypothesized that Nrf2 acts via a mechanism involving the expression of heme oxygenase-1 (HO-1/*Hmox1*) gene, which inhibits the pathologic outcome of autoimmune neuroinflammation.

Materials and methods: C57BL/6 *Nrf2*-deficient ($Nrf2^{-/-}$), myelin oligodendrocyte glycoprotein peptide 35−55 (MOG_{35-55}) specific T cell receptor transgenic (2D2) crossed with $Nrf2^{-/-}$ and $Nrf2^{+/+}$, IFN-g receptor-deficient ($Ifngr1^{-/-}$) mice and *CD11c-Cre* crossed with $Hmox1^{lox/lox}$ were used. For EAE induction, mice were immunized with MOG_{35-55} (100 mg) emulsified in CFA (100 ml; 4 mg/ml) subcutaneously on each side of the lower abdomen and received Pertussis toxin (PTx) intravenously (200 ng in PBS; 100 ml) after immunization (i.e. 4h and 2 days). 2D2$Nrf2^{+/+}$ and 2D2$Nrf2^{-/-}$ mice received PTx intravenously (200 ng in PBS; 100 ml) twice at 48h interval. Clinical signs of experimental autoimmune encephalomyelitis (EAE) were evaluated daily for 40 days and scored as described elsewhere. For the IL-12/23p40 *in vivo* neutralization assay, 2D2$Nrf2^{+/+}$ and 2D2$Nrf2^{-/-}$ mice received PTx intravenously (200 ng in PBS; 100 ml) twice with a 48h interval. At day-1 and 6 post-PTx administration, mice received either anti-IL12/23p40 neutralizing antibody (C17.15, 1 mg/mouse) or a rat isotype-matched IgG2a control antibody (YKIX-302; 1 mg/mouse) i.p. dissolved in PBS.

Results: When immunized withMOG_{35-55}, $Nrf2^{-/-}$ mice develop a more severe form of EAE, as compared to wild type ($Nrf2^{+/+}$) controls. This is also the case for 2D2 $Nrf2^{-/-}$ versus $Nrf2^{+/+}$ mice, without MOG_{35-55} immunization. The protective effect of Nrf2 is exerted in leukocytes, as demonstrated by the adoptive transfer of leukocytes from MOG_{35-55}-immunized 2D2 $Nrf2^{-/-}$ versus $Nrf2^{+/+}$ mice into naïve recipients. Expression of Nrf2 in DC inhibits IL-12 production, revealed by increased IL-12 expression in $Nrf2^{-/-}$ versus $Nrf2^{+/+}$ DC. This favors myelin-reactive T helper (T_H) cell priming towards a T_H1 phenotype secreting IFN-g. Inhibition of IL-12, using a neutralizing anti-IL-12/23p40 monoclonal antibody, reverts this effect and suppresses the development of EAE in 2D2 $Nrf2^{-/-}$ or $Nrf2^{+/+}$ mice. This is also the case when the IFN-g biologic activity is suppressed in $Ifngr1^{-/-}$ mice. Specific deletion of the *Hmox1* allele in DC enhances IL-12 secretion by DC as well as T_H1 cell priming and EAE severity in MOG_{35-55} immunized *CD11c-Cre/*$Hmox1^{-/-}$ versus $Hmox1^{lox/lox}$ mice.

Conclusions: In conclusion, we propose that Nrf2 counters the pathogenesis of autoimmune neuroinflammation via an immunoregulatory mechanism exerted in DC and involving the expression of HO-1.

P0067

Tim-3 and PD-1 are differently expressed on exhausted T cells in HIV infected patients

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Purpose/Objective: Progressive loss of T cell function is an important mechanism of chronic HIV-1 infection. PD-1 has been primarily used to describe exhausted T cells. Recently Tim-3 has been identified as additional marker for dysfunctional T cells in chronic virus infections. Tim-3 positive cells were reported to differ from PD-1 positive T cells, but these cells have not been defined in detail.

Materials and methods: In this study we have investigated the expression of PD-1 and TIM-3, two markers implicated in immune exhaustion on T cells from HIV-1 infected individuals.

Results: Interestingly we found that even in the viremic patients only a small percentage of T cells expressed Tim-3 (mean: 4.1%). In contrast, a significant amount of T cells were PD-1 positive (mean 36.8%) and furthermore PD-1 was expressed at much higher levels than Tim-3. Nevertheless we found a trend to higher numbers of Tim-3 positive cells in viremic than in aviremic HIV infected and even lower numbers in healthy individuals. When analysing CD8 T cells of viremic and aviremic patients regarding CD45RA expression we found a striking difference between Tim-3 and PD-1 positive T cells: the Tim-3 expressing cells were found in the CD45RA positive subset ($P < 0.5$) whereas PD-1 expression was nearly exclusively found on the CD45RA negative subset ($P < 0.5$). To further characterize the Tim-3 positive subset we performed multicolour staining using antibodies to various T cells markers including CD28 and CD57. Although CD57 expression and loss of the CD28 molecule are both associated with T cell senescence, Tim-3 expressing cells were exclusively found in the CD57 positive subset whereas is was not restricted to CD28 negative T cells.

In contrast to the PD-L/PD-1 pathway, blockingTim-3 did not enhance HIV specific T cell proliferation or IFN g secretion.

Furthermore, whereas PD-1 positive cells have previously been shown to be increased in HIV infected patients with discordant immune response, Tim-3 expression did not differ between patients with or without immune restoration on HAART.

Conclusions: Taken together our data implicate that Tim-3 defines a novel subset of terminally differentiated T cells.

Poster Session: Innate Mucosal Immunology

P0068

A role for the pattern recognition receptor Nod2 in recruitment of CD103$^+$ DC to the gut in helminth infection

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Purpose/Objective: The ability of the colon to generate an immune response to pathogens, such as the whipworm *Trichuris muris*, is a fundamental and critical defence mechanism. Our previous work demonstrated that resistance to infection is associated with the rapid recruitment of dendritic cells (DCs) to the colonic epithelium via epithelial production of CCL5 and CCL20. However, the epithelial-parasite interaction that drives chemokine production is not known. Here, we address the role of the cytosolic pattern recognition receptor Nod2, the location of which within the crypts correlates with the *T.muris* niche.
Materials and methods: *Nod2*$^{-/-}$ and WT mice were infected with T.muris and DCs in the large intetsine anlaysed by flow cytometry and immunohistochemistry. DC migration was analysed using bone marrow chimeras and chemotaxis assays. Epithelial chemokine production was assesed by qPCR and ELISA.
Results: There was a rapid influx of CD103$^+$ CD11c$^+$ DCs into the colonic epithelium in WT mice whereas DC recruitment was impaired in *Nod2*$^{-/-}$ animals. Strikingly, the number of colonic CD11c$^+$CD103$^+$ DCs in *Nod2*$^{-/-}$ mice remained low until D7 post-infection. Macrophage recruitment was unaffected. Migration assays revealed no difference between the migration of *Nod2*$^{-/-}$ and WT colonic DCs in response to chemokines. Furthermore, bone marrow chimeras of wildtype mice reconstituted with *Nod2*$^{-/-}$ cells unequivocally demonstrated that *Nod2*$^{-/-}$ DCs recruitment was restored in the presence of a WT epithelium. *In vivo* and *in vitro* experiments showed that epithelial production of chemokines, CCL2, CCL3 and CCL5 by *Nod2*$^{-/-}$ epithelial cells was markedly reduced. In addition, Nod2$^{-/-}$ was upregulated in WT epithelial cells 24 h pos-infection.
Conclusions: Collectively, these data indicate a role for Nod2 in recognition of the helmith *T.muris* via epithelial chemokine production and recruitment of CD103$^+$ DCs to the colonic epithelium.

P0070

An investigation of the capacity of colonic macrophages to proliferate in CX3CR1$^+$ /eGFP mice infected with the parasitic nematode *Trichuris muris*

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Purpose/Objective: *Trichuris muris* is a nematode parasite of the mouse which dwells in the large intestine. It is a natural mouse model of *Trichuris trichiura*: a prevalent and debilitating parasite of humans worldwide. A Th2 immune response is essential for the expulsion of worms. However, the nature of the ensuing inflammatory response (and its regulation) is not fully understood. Previous studies in this laboratory have shown that macrophages are the predominant type of inflammatory cell in the large intestine post-infection. Recently, macrophages have been shown to proliferate during certain inflammatory responses. This study aims to determine the capacity of colonic macrophages to proliferate (by analysing the incorporation of BrdU into DNA) during infection with *T. muris*.
Materials and methods: Mice were injected i.p. with BrdU. Four hours later, leukocytes were isolated (by enzymatic digestion) from the large intestine of CX3CR1$^{+/eGFP}$ mice and the macrophages were analysed by multi-colour flow cytometry. In this mouse, cells expressing the chemokine receptor CX3CR1 also express eGFP.
Results: Two contrasting populations of CX3CR1$^+$ macrophages were identified. The first, F4/80^{high}CX3CR1^{high} and predominantly Ly6C$^-$, was relatively abundant in uninfected mice. This phenotype is consistent with resident macrophages. In contrast, the second, F4/80^{low}CX3CR1^{low} and Ly6C$^+$ was relatively abundant post-infection. This population is consistent with inflammatory macrophages. Less than 7% of either population of macrophages expressed RELMa, a marker of alternative activation, and there was no significant difference post-infection. In naïve mice, <2% of the resident or inflammatory macrophages had incorporated BrdU into their DNA. However, there was a twofold increase post-infection, but only in the resident macrophages. A relatively high proportion (approximately 30%) of these BrdU$^+$ resident macrophages also expressed RELMa.
Conclusions: In contrast to other models of inflammation, only a small proportion of colonic macrophages proliferates during *T. muris* infection. These cells were resident-type macrophages, some of which were alternatively activated. Compared to the accumulation of inflammatory macrophages derived from blood monocytes, the proliferation of resident macrophages makes only a small contribution to the inflammatory response.

P0072

Bladder-resident phagocytes in the response to luminal bacteria

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Purpose/Objective: The bladder is a unique mucosal barrier devoid of microbiota. The most prevalent diseases of the bladder are urinary tract infection (UTI and bladder cancer. One common feature between the diseases is the introduction of bacteria into the lumen, uropathogenic *E. coli* (UPEC) in the case of UTI or BCG as immunotherapy for bladder cancer. UPEC and BCG instillation into the bladder have different outcomes, where BCG induces CD8$^+$ T cell cross-priming, while immunity to UPEC appears to be primarily antibody-mediated. We hypothesized that the basis for this difference is due in part to differential bacterial uptake by phagocytes in the bladder mucosa.
Materials and methods: To assess uptake by bladder phagocytes, we developed a fluorescent particulate model antigen coated with bacterial adhesin to overcome the complication that material instilled intravesically is quickly expelled during micturition. We employed flow cytometric analysis, immunohistochemistry, and functional assays to investigate uptake following instillation of model antigen, UPEC, or BCG in mouse models of urinary tract infection and BCG immunotherapy.
Results: We characterized four subsets of dendritic cells (DCs) and macrophages resident in naïve bladders. We observed that model antigen was taken up by bladder-resident macrophages. By contrast, UPEC, which induces bladder cell exfoliation, was phagocytized by macrophages and CD103$^+$ DCs. As the number of BCG in the bladder is significantly reduced compared to UPEC, we used a functional assay to measure uptake, observing that CD86 expression was increased on the surface of CD11c$^+$ cells enriched from the bladder and these cells presented antigen. Interestingly, despite very low CFU in the bladder,

BCG disseminated to the lymph nodes and spleen of treated mice while UPEC remained bladder-localized.
Conclusions: These studies suggest that different bladder APC populations take up UPEC and BCG, perhaps ferrying BCG to distal sites of the host, resulting in distinct immune responses. This may be due to bacteria localization in relation to resident APC. These findings contribute to the understanding of the disparity in adaptive immune responses initiated by BCG or UPEC, which may translate to mechanisms to enhance clearance of UPEC and improve the efficacy of BCG immunotherapy for bladder cancer.

P0073

$CD161^{++}CD8^{+}$ T cells express IFNgamma in response to TLR8 stimulation in an IL-12^{+} IL-18 dependent, TCR independent, manner

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Purpose/Objective: The high expression of CD161 is a characteristic of the human mucosal-associated invariant T (MAIT) cells / Tc17 subpopulation. T cells expressing the invariant V-alpha7.2 TCR make up ~85% of the adult $CD161^{++}CD8^{+}$ T cell population. A further characteristic of this population is the high expression of the IL-18 receptor, which correlated with CD161 expression in T cells. This suggested that these cells were potentially strong responders to IL-18 and IL-12 stimulation, which has long been known to induce IFN-gamma in both NK and T cells in a TCR independent manner.
Materials and methods: Human peripheral blood mononuclear cells (PBMC) from healthy individuals were stimulated with IL-12 and/or IL-18, TLR agonists. Cytokine expression by $CD161^{++}CD8^{+}$ T cells was assessed by FACS.
Results: Here we show that in humans $CD161^{++}CD8^{+}$ T cells were the primary T cell population to respond to IL-12^{+} IL-18 stimulation, without prior activation or CD3 cross-linking. This response was restricted to IFN-gamma secretion, which was delayed compared to TCR triggering, although the delay was reduced with prior IL-12 priming, and was enhanced by IL-15 co-stimulation. TLR8 agonist stimulation of PBMCs resulted in IFN-gamma production specifically by the $CD161^{++}CD8^{+}$ T cell and NK cell populations. This response could be blocked by either anti-IL-12 or anti-IL-18 antibodies, or inhibition of TLR8 or caspase-1. This is of particular interest as MAIT-cells have been previously described to be restricted to an as-yet unidentified bacterially derived ligand/s, presented on the MHC-like protein, MR1.
Conclusions: These data show, for the first time, that $CD161^{++}CD8^{+}$ T cells are the major T cell population that responds to directly IL-12^{+} IL-18 stimulation in humans without TCR triggering. Moreover, $CD161^{++}CD8^{+}$ T cells could also respond rapidly *in vivo*, in a TCR independent fashion, to cytokines, and potentially, via TLR8, to viral infections by single-stranded RNA viruses, such as HIV, HCV or Dengue.

P0074

Characterization of tonsil infiltration and gene expression profile of innate sensors in PFAPA patients

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Purpose/Objective: Periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis (PFAPA) syndrome is the most common periodic fever disease in young children classified in the group of autoinflammatory syndromes. The etiology of this disorder is still unknown. Palatine tonsils are sites where innate immunity leads to onset of the adaptive immunity, mediated by B and T lymphocytes. Three families of pathogen sensors mediate the recognition of microbes: Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). We aimed to investigate differences in leucocytes subpopulations and innate receptors gene expression of palatine tonsil cells from patients with PFAPA in order to understand the pathogenesis of this inflammatory condition.
Materials and methods: We have collected tonsil tissue from two groups of pediatric patients undergoing tonsillectomy: PFAPA patients whose genetic testing excluded hereditary periodic fevers (HPFs) during flares and asymptomatic intervals ($n = 20$), and patients undergoing to tonsillectomy due to recurrent bacterial tonsillitis (control group, CG) ($n = 16$). We have performed staining of subpopulations on tonsil cells and tissues using flow cytometry and immunohistochemistry. We have analyzed TLRs, NLRs, and RLRs gene expression by quantitative real-time RT-PCR.
Results: The histology of tonsils in PFAPA patients shows a preservation of tonsillar architecture without specific chronic inflammation with respect to CG. FACS analysis demonstrate a higher number of naïve and a significantly lower percentage of effector memory $CD4^{+}$ and $CD8^{+}$ T cells in PFAPA patients compared to CG. Moreover, we observe a significantly recruitment of NK cells in PFAPA patients with respect to CG. Tonsil cells express a broad repertoire of TLRs variably represented in both PFAPA and CG. Of note, in PFAPA patients we discover a significant increase in the gene expression of NALP1 and NALP3 and higher levels of RIG-1 expression when compared to CG.
Conclusions: In summary, these results indicate a possible involvement of NK cells and of innate receptors (NLRs and RLRs) in pathogenesis of PFAPA supporting the crucial role of the innate immunity. Nonetheless, the high numbers of undifferentiated naïve T cells in PFAPA patients suggest that adaptive immune responses might *be implicated in these autoinflammatory* disorders.

P0075

COX-2 Modulates IL-36γ Expression by HPV6/11^{+} laryngeal keratinocytes' in patients with recurrent respiratory papillomatosis

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Purpose/Objective: Determine why HPV6/11 chronically infected laryngeal keratinocytes (LKs) from recurrent respiratory papillomatosis (RRP) patients that over-express COX-2 and IL-36γ fail to activate resident, immature Langerhans cell (LCs) in papillomas.

Materials and methods: Affymetrix HG-133A gene expression arrays identified differential expression IL-36g between papillomas and unaffected autologous laryngeal tissues from RRP patients ($P < 0.001$, paired t-test), and COX-2 was over expression by both tissues. Cultured HPV^+ papilloma cells or HPV^- LK from patients were studied for IL-36γ expression by qPCR and western analysis, after IL-36β or IL-36γ treatment,$^{+/-}$COX-2 inhibition (celecoxib, 5 μM) and$^{+/-}$ 500 pg/ml PGE_2. IL-36γ release was detected by ELISA (Adipobiotech).
Results: We asked if IL-36γ stimulation of HPV^+/HPV^- laryngeal cells affected mRNA expression of IL-1β, TNF-α or IL-36γ following by IL-36γ itself, and if pretreatment with celecoxib that eliminate PGE_2 effects secretion of these proinflammatory cytokines. LK IL-36γ treatment showed little effect on HPV^+/HPV^-LK IL-1β expression. TNF-α was elevated in untreated papilloma cells, versus HPV^- cells, and was increased in IL-36γ treated HPV^-, and more so in papilloma LKs ($P < 0.05$).Basal and IL-36γ-stimulated TNF-α levels were suppressed by celecoxib pretreatment.Thus, PGE_2 enhances TNF-α expression in LKs. However, IL-36γ treatment of HPV^- LKs showed a 14-fold increase in IL-36γ after IL-36γ exposure ($P = 0.03$), and greater in LKs pretreated with celecoxib (46.6-fold; $P = 0.0003$). IL-36γ treated papilloma cells increased IL-36γ expression 15-fold ($P = <0.01$), similar to HPV^- LKs.IL-36γ expression by papilloma cells was 20-fold higher to begin with ($P = 0.006$). Pretreatment with celecoxib increased IL-36γ in HPV^- LKs treated, but did not further upregulate IL-36γ in papilloma LKs, possibly because IL-36γ expression was so high after IL-36γ treatment. However, IL-36γ was not found in culture medium.We also tested monocyte-derived Langerhans cell (iLCs) responses to IL-36γ; they expressed IL6, IL-12p40.
Conclusions: HPV infection drives very high levels of IL-36γ expression in HPV^+ LKs. PGE_2 modulates this.While celecoxib decreased IL-36γ-induced TNF-α expression, it increased IL-36γ expression in HPV^+ LKs. Thus, IL-36γ has a positive feed-back loop. IL-36γ release by RRP-derived LKs is not spontaneous.A secondary signal may be needed, such as poly(I:C)/another Toll-like signal/ATP/IL-17. Control LKs are under study to determine if they can release IL-36γ. IL-36 papilloma cells/HPV^+ cells can mature iLCs and support effective adaptive anti-HPV immunity.

P0076
Critical role of type III interferons during rotavirus infection

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Purpose/Objective: Type I and type III interferons (IFN) bind to different cell surface receptors but induce identical signal transduction pathways leading to the expression of antiviral host effector molecules. Although IFN-λ has been shown to predominantly act on mucosal organs such as the lung and gastrointestinal tract, studies using IFN-λ receptor-deficient mice have failed to define a non-redundant function. Here we analyse the role of IFN-λ during infection with an epithelial tropic enteric virus to elucidate the contribution to antiviral protection at mucosal surfaces.
Materials and methods: IL28Rα-deficient, IFNAR-deficient and IL28Rα/ IFNAR-double deficient mice were analysed during oral rotavirus infection. Viral titer in the intestine was determined by ELISA. The response of the intestinal epithelium was analysed by quantitative PCR and simultaneous immunostaining of virus-infected cells and the antiviral Mx1 protein were performed.
Results: Reduced expression of IFN response genes were found in type III interferon signalling deficient mice in contrast to IFNAR-deficient or wild type mice. In accordance, enhanced virus replication and tissue damage was found in the absence of IFN-λ signalling, whereas no significant contribution of IFN-α/β signalling to the antiviral host defence was detected.
Conclusions: These findings identify a critical role of IFN-λ to induce an antiviral response and to restrict virus replication in the intestinal epithelium.

P0077
Dectin-1 is required for control of systemic, but not gastrointestinal, infections by *Candida albicans*

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Purpose/Objective: *Candida albicans* is a human fungal commensal, commonly colonising the gastrointestinal tract. However, *C. albicans* can cause both mucosal and systemic infections if the host immune response becomes compromised. In mice and humans the innate pattern recognition receptor Dectin-1 is involved in the control of fungal infections. However, the role of Dectin-1 during colonisation of the gastrointestinal tract remains to be defined. Here we investigated the role of Dectin-1 in systemic candidiasis as well as mucosal colonisation of the gastrointestinal tract following oral infection.
Materials and methods: For systemic infections, mice (C57/BL6 wild-type and Dectin-$1^{-/-}$) were infected I.V. with 2×10^5CFU *C. albicans* SC5314 via the lateral tail vein. For colonisation of the gastrointestinal tract, mice were pre-treated with antibiotics before exposure to 1×10^7 CFU/ml *C. albicans* SC5314 (for 5 days) in the drinking water. Colonisation was maintained using antibiotics and monitored via stool fungal burdens. Mice were analysed at various time points for tissue fungal burdens, cytokine responses and pathology via histology.
Results: Dectin-1 deficiency correlated with increased fungal burdens and dysregulated cytokine production in gastrointestinal tract tissues during systemic infection. Surprisingly, following oral infection via the drinking water, there was no Dectin-1 involvement in inflammation, fungal burdens or cytokine responses to GI colonization with *C. albicans*.
Conclusions: Here we demonstrate in mice that Dectin-1 is essential for controlling dissemination to the tissues of the gastrointestinal tract during systemic infection with *C. albicans*; however this receptor appears to be redundant for controlling colonisation of the gastrointestinal tract following oral infection.

P0078
Deviant expression of defensins in sera and saliva of patients with autoimmune polyendocrine syndrome type I

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Purpose/Objective: Patients with autoimmune polyendocrine syndrome type I (APS I) have high titer neutralization IgG autoantibodies against Th17-expressed cytokines. Autoantibodies against IL17F and IL22 are present in up to 90% of APS I patients, and their presence in sera correlate to chronic *Candida* infections. We have further previously reported that APS I patients exhibit lower secretion of IL17F and IL22 than healthy controls. In an attempt to elucidate the mechanisms behind these phenomena, we have characterized the IgA and IgG autoantibody profiles against Th17-expressed proteins in sera and saliva from APS I patients and controls. Since especially IL22 can influence the expression of antimicrobial peptides (AMPs), which mediates the destruction of invading pathogens (including *Candida*),

we have also examined the expression of key set of key AMPs and anti-fungi agents in sera and saliva from APS I patients and controls.
Materials and methods: We have measured IgA and IgG antibodies aganst Th17-mediators in sera and saliva using RIA and an in-house ELISA. We have further examined the expression of alpha-defensins (HNP 1−3), beta-defensin 1, 2 and 4, calprotectin, LL-37, Bactericidal permeability increasing protein and Myeloperoxidase in saliva and sera from Norwegian APS I patients and healthy controls using commercial ELISA.
Results: We confirm the high prevalence of serum IgG autoantibodies against IL17F and IL22 in APS I patients. A significantly higher level of IgA autoantibodies against IL22 were also found in both sera and saliva from these patients compared to healthy blood donors. This kind of response could be important in mucosal immunity and prone APS I patients to resistance against Candida clearance. Our results further indicate an imbalance of both alpha-defensins and beta-defensins in APS I patients compared to healthy controls. Importantly, the actual profile of autoantibodies and AMP expression varied a lot between patients.
Conclusions: We conclude that a high level of IgG and/or IgA autoantibodies against cytokines involved in AMP expression could be correlated to a deviant level of AMPs, which again may mediate chronic candidiasis. Further studies will be necessary to confirm our findings.

P0079
Differential functional contributions of monocyte descendants to gut homeostasis and inflammation as effector monocytes, macrophages and dendritic cells

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Purpose/Objective: Based on specific growth and transcription factor requirements, as well as results of adoptive precursor transfers, mononuclear phagocytes are currently divided into two sublineages, with MDPs (i) giving rise to dedicated precursors of classical Flt3L-dependent dendritic cells and (ii) differentiating into monocytes that are called to sites of inflammation to yield macrophages.The healthy intestinal lamina propria is unique in that it constantly recruits Ly6C^{hi} monocytes, presumably attracted by tonic low-grade inflammation resulting from exposure to the commensal microflora. Once extravasated monocytes give rise to resident macrophages that contribute with their distinct non-inflammatory gene expression signature critically to maintain gut homeostasis.
Materials and methods: Our study uses DSS-challenged mice as colitis model and employs adoptive monocyte transfers, flow cytometry and gene expression analysis and and two photon imaging to establish monocyte fates in the inflamed colon.
Results: Here we report on monocyte fates in the inflamed colon. Specifically we show that infiltrating Ly6C^{hi} monocytes gain in this tissue context effector functions that render them responsive to bacterial products and as a result pro-inflammatory. Moreover, monocyte ablation established these cells as critical drivers of acute DSS-induced gut inflammation. Interestingly though, with time monocytes differentiate into migratory antigen-presenting cells, reminiscent of dendritic cells.
Conclusions: Collectively, our results highlight cellular dynamics in the inflamed colon and the plasticity of Ly6C^{hi} monocytes singling them out as potential targets for IBD therapies.

P0081
Enteropathogenic *Escherichia coli* engages in temporal regulation of the inflammasome by interaction with NLRC4 and NALP3

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Purpose/Objective: Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of acute and chronic diarrhoea in developing nations, principally affecting children <2 years of age. The interaction of EPEC with the gastrointestinal (GI) mucosal immune system remains largely uncharacterised. A recent study by Lebeis *et al* 2011 indicated a critical role for IL-1 receptor signalling in *Citrobacter rodentium* (murine model for EPEC) mediated disease pathogenesis. In the present study we investigated the signalling events that regulate IL-1β production in response to EPEC infection. Better understanding of this complex pathway may highlight novel therapeutic targets for infection control and highlight strategies for vaccine development for this major enteropathogen.
Materials and methods: Murine Bone Marrow derived Dendritic cells (BMDC) from mice lacking various inflammasome components (NLRC4$^{-/-}$, NALP3$^{-/-}$, ASC$^{-/-}$, CASPASE$^{-/-}$) were infected with EPEC Wild Type (WT) E69 strain and isogenic mutant strains deficient in the flagella and structural and effector protein(s) of the T3SS (Type III secretion system). Inflammasome activation was assessed by gene and protein expression, flow cytometry and confocal microscopy.
Results: WT E69 strain induced marked increase in bioactive IL-1β in a caspase-1 dependant manner. This effect required the presence of the T3SS component in EPEC. In contrast to published work by Miao *et al* 2010, we found a minimal role for flagella-mediated IL-1β as ΔFliC a mutant strain lacking the flagella elicited IL-1β to levels seen during infection with WT strain in NLRC4$^{-/-}$ DC. ASC (an essential adaptor required for regulating mature caspase-1 by the inflammasome) speck formation and IL-1β secretion was significantly decreased in NLRC4$^{-/-}$and NLRP3$^{-/-}$suggesting involvement of both proteins in ASC mediated caspase-1 activation.
Conclusions: An intact T3SS was found to be necessary for optimal IL-1β release. Our experiments indicate potential role of ASC in EPEC-mediated IL-1β release especially in the presence of the inflammasome NLRC4. Our study is the first to highlight temporal involvement of NLRC4 and NALP3 in EPEC-mediated inflammasome activation.

P0082
Epithelial responses to pathogenic or commensal oral biofilms

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Purpose/Objective: Periodontal disease is the result of chronic inflammation mediated by the host immune response to bacterial biofilm (plaque) on the root surface, leading to destruction of supporting structures of the teeth and ultimately tooth loss. In health, commensal oral biofilms form without notable disruption of the tooth supporting tissues. However, introduction of 'keystone pathogens' such as *P. gingivalis* creates a pathogenic biofilm which evokes a damaging inflammatory response with local tissue destruction.

To compare the response of oral epithelial cells to pathogenic and commensal biofilms and investigate the mechanisms by which pathogenic species alter the epithelial cell inflammatory response *in vitro*.
Materials and methods: OKF6-TERT2 oral epithelial cells were stimulated with different species of bacteria (*S. mitis, P. gingivalis, F. nucleatum and A. Actinomycetemcomitans*). Bacterial stimulations were prepared as planktonic bacteria, mixed or single species biofilms.

Changes in mRNA and protein expression of multiple chemokines and cytokines were then assessed by Taqman® low density array or ELISA.
Results: Significant differences between epithelial cell cytokine and chemokine in response to pathogenic or commensal biofilms were observed at both protein and mRNA level. *P. gingivalis* degraded IL-8 and demonstrated strain dependent variation in epithelial cell stimulation. These results demonstrate that oral bacteria may modulate the epithelial cell response to allow persistence of the biofilm.
Conclusions: Periodontal disease associated bacteria are able to modulate epithelial cell chemokine and cytokine expression both at a gene and protein level. The subversion of the host response, such as cytokine degradation by *P. gingivalis,* may contribute to the chronicity of infection and subsequent progression of periodontal disease.

P0083
Essential role for type I interferons in the regulation of T cell mediated colitis

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Purpose/Objective: We explored the role of type I interferons (IFN-1) in the regulation of intestinal homeostasis.
Materials and methods: We used the T cell adoptive transfer model of colitis to study intestinal homeostasis *in vivo*. We also isolated various cell populations from the colon lamina propria to study the dependence of their functions on type I interferon by employing methods of *in vitro* stimulation.
Results: Colon mononuclear phagocytes (MP) constitutively produced IFN-1 that was dependent on TRIF but not MyD88-signaling. Transfer of $CD4^+$ $CD45RB^{hi}$ T cells from wild type (WT) or interferon a/b receptor-deficient ($IFNAR1^{-/-}$) mice into $RAG^{-/-}$ hosts resulted in similar colitis development. In contrast, $RAG^{-/-}$/$IFNAR1^{-/-}$ double knockout (DKO) mice developed accelerated severe colitis compared to $RAG^{-/-}$ hosts transferred WT $CD4^+$ $CD45RB^{hi}$ T cells. Enhanced colitis was associated with increased T cell proliferation in the mesenteric lymph nodes and subsequent accumulation in the colon lamina propria and was suppressed by IFNAR signaling on host hematopoietic cells. Additionally, blockade of IFN-1 by a neutralizing monoclonal antibody against IFNAR1 resulted in exacerbated colitis. Finally, we show that colon mononuclear phagocytes (MP) from $IFNAR1^{-/-}$ mice produced less IL-10 than from WT mice upon *ex vivo* stimulation.
Conclusions: These data are the first to demonstrate an essential role for IFN-1 in controlling the production of IL-10 by gut MP and the indirect maintenance of intestinal T cell homeostasis.

P0084
Ex vivo imaging of free radicals reveals robust immune activity in the distal small intestine in NOD mice

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Purpose/Objective: Environmental influence on autoimmunity in type 1 diabetes is thought to be ascribed to dietary and microbial factors, most of which affect the gastrointestinal tract. In the nonobese diabetic (NOD) mouse, signs of enteropathy and increased immune activity are present in both small and large intestine.
Materials and methods: We evaluated inflammatory activity in the whole gut in prediabetic NOD mice by *ex vivo* imaging of reactive oxygen intermediates (ROI) with a chemiluminescent probe and In-vivo imaging system (IVIS), cytokines and bacterial 16s RNA were measured using QPCR and cells were characterised with flow cytometry.
Results: Young NOD mice displayed high ROI activity in the distal small intestine, ileum. *In vivo* staining with dihydroethidium revealed that the major ROI producers were intestinal epithelial cells. Anti-diabetogenic casein hydrolysate diet diminished ROI activity, expression of IL-17 and proinflammatory cytokines, T-cell activation and total bacterial load in ileum. Despite all this, gut permeability to oral FITC-dextran was not reduced.
Conclusions: Our findings suggest that secondary to an underlying permeability disorder, diet-dependent bacterial colonisation induces epithelial ROI production and local T-cell activation in the ileum of NOD mice. These findings establish a new link between diet, microbial factors and dysbalanced gut homeostasis in the NOD mouse, with possible implications for the immune pathogenesis of type 1 diabetes.

P0085
Function and differentiation of intestinal macrophages in oral tolerance

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Purpose/Objective: $CD11b^{-/-}$ and $CX3CR1^{-/-}$ mice fail to establish oral tolerance. This defect can be rescued by the adoptive transfer of wild type macrophages, suggesting a central role of intestinal macrophages in intestinal tolerance generation. We have previously shown that CX3CR1 drives the Interleukin-10 (IL-10) secretion by gut resident macrophages and thereby the local expansion of regulatory T (Treg) cells. Here we analysed the role of CD11b in intestinal tolerance induction and macrophage differentiation.
Materials and methods: The myeloid compartment of $CD11b^{-/-}$ mice was compared to wild type mice using flow cytometry and iterative Chipbased Cytometry (iCBC). Cytokine production by intestinal macrophages was analysed and adoptive transfer experiments were performed to follow Treg induction and expansion *in vivo*. The differentiation of intestinal macrophages was studied by photoconversion and iCBC.
Results: Treg were readily induced in the gut draining mesenteric lymph nodes of wild type and CD11b-deficient mice. In contrast, the local expansion of Treg was impaired in the absence of CD11b. CD11b deficient mice showed a wild type-like composition of intestinal myeloid cells and gut resident macrophages secreted normal amounts of IL-10. To track the local differentiation from incoming progenitors to tolerogenic $CX3CR1^{high}CD11b^+$ macrophages, we compared the myeloid compartment as a function of local residence time in the gut lamina propria by photoconversion and iCBC.
Conclusions: The expression of CX3CR1 and CD11b affect the expansion of Treg cells in the gut lamina propria. Yet, in contrast to CX3CR1, the lack of CD11b does not affect the cytokine profile of intestinal macrophages. Elucidating the differentiation process of intestinal macrophages, its regulation in inflammation and steady state and the function of CX3CR1 and CD11b will help for the further understanding of oral tolerance.

P0086

Generation and phenotypic analysis of murine beta defensin knockout mice

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Purpose/Objective: Defensins are thought to play a role in the protection against infection and immunological homeostasis, however their role is not completely characterised. Experimental induced mutations of defensin genes may enhance our understanding of their microcidal and immunological activity. We have generated mice that lack murine beta defensin 14 (mBD14), which was recently identified as the orthologue of human beta defensin 3 (HBD3). As the defensin genes are in close proximity to one another, we used oligonucleotide-mediated gene modification (oligo targeting), which created a specific mutation by inserting a 4bp sequence in the *Defb14* gene creating a stop codon and a frameshift. This mouse mutant was then phenotypically analysed.
Materials and methods: HBD3 has been associated with ulcerative colitis, as patients with ulcerative colitis exhibit increased expression of HBD3 mRNA. Therefore the ability of mBD14 to maintain the immunological homeostasis of the gut, was investigated by DSS induced colitis. Additionally, to determine if the microbiota of the mBD14$^{-/-}$ mice is different to WT and mBD14$^{+/-}$ mice, a 16s rRNA PCR was performed and PCR products were run on a denaturing gradient gel electrophoresis (DGGE) gel.
Results: The mutation in the *defb14* gene has been confirmed in the homozygous mice at the genomic, RNA and protein level. There was no difference in the severity of colitis in the colon of the mBD14$^{-/-}$ mice compared with mBD14$^{+/-}$ mice. Additionally, no difference in the DGGE profiles of caecal contents and skin were observed for WT, mBD14$^{+/-}$ and mBD14$^{-/-}$ mice.
Conclusions: No phenotype was established for the mBD14$^{-/-}$ mice, therefore further defensin genes (*Defb18*, *Defb41*, *Defb7*, *Defb40*, *Defb20*) will be inactivated by TALEN technology, to create single, double and defensin cluster mutants. These mutants will be phenotyped with the same methods used to analyse the mBD14 mutant mice. These experiments may help clarify the role of defensins in immunological homeostasis.

P0087

Humoral and cellular immunity to food antigens in patients with leukemia

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Purpose/Objective: Patients with food allergy have well known abnormal immune response to various food antigens. Abnormal and dysfunctional response of immune cells may represent a predisposition for the development of hematological malignancies, especially leukemia. Chronic stimulation of dysfunctional immune cells in leukemia patients with some antigen that is constantly present in a daily diet can lead to increased number of malignantly transformed cells. The aim of this study was to determine the humoral immunoreactivity to food antigens (gliadin, cow's milk proteins – CMP and phytohaemagglutinine – PHA) and cellular immunoreactivity to those antigens in leukemia patients.
Materials and methods: Study involved 35 patients with different types of leukemia. The control group consisted of 72 healthy volunteers. The Home Made ELISA test to gliadin, CMP and PHA were done to determine humoral immunity. For the assessment of cellular immunity PBMC were isolated and incubated in RPMI with 10% autologous plasma without and with different food antigens: gliadin, CMP and PHA. Index of PBMC stimulation was determined using MTT test. Cut-off for both tests, ELISA ant MTT, was determined as mean ($X \pm 2SD$)%.
Results: The most pronounced stimulation of PBMC was detected with gliadin 12/35 patients had increased growth of PBMC. Among them, 2 had enhanced IgA immunoreactivity to gliadin, one patient had increased IgG immunoreactivity and 3/12 had increased IgE immunoreactivity. Only one patient had suppression of growth of PBMC with gliadin but without increased humoral immunoreactivity to tested antigens. 2/35 patients had suppression of growth of PBMC with CMP and both of them had enhanced IgA immunoreactivity to CMP. 11/35 patients had suppression of growth of PBMC with PHA, amongst them three patients had enhanced IgA immunoreactivity to PHA and one had enhanced IgM immunoreactivity to PHA.
Conclusions: These preliminary results showed that enhanced humoral immunoreactivity to some of the tested antigens is present together with the suppression of growth of PBMC to the same antigen in some of the examined leukemia patients. This point to the need to elucidate clinical importance of those findings in these particular leukemia patients, i.e. to give the answer could stimulation of mucosal immunity with food antigens affect the survival of the malignant cell clone.

P0088

IL-17E: a negative regulator of periodontal inflammation?

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Purpose/Objective: IL-17E (IL-25) has a key role in promoting Th2-driven pathologies but has also been demonstrated to down regulate destructive localised inflammatory responses in conditions such as rheumatoid arthritis. Periodontal disease is a chronic inflammatory disease which left untreated leads to tooth loss. At present the role of IL-17E in periodontal disease pathogenesis is completely unknown. Therefore, the purpose of this study was to investigate the expression of IL-17E within the periodontoium and begin to delineate the role IL-17E may play in the pathogenesis of periodontal disease.
Materials and methods: Real time PCR was employed to compare the expression of IL-17E mRNA in healthy and diseased periodontal tissue. The cell types within the periodontium responsible for expression of IL-17E and its receptors (IL-17RA and IL-17RB) were then determined by immunohistochemistry. Using a live biofilm of the periodontal pathogen, *P. gingivalis*, combined with ELISA technologies and real time PCR, the ability of IL-17E to modulate the oral keratinocyte immune response was then investigated *in vitro*.
Results: Real time PCR analysis showed that IL-17E mRNA levels are up-regulated in periodontal disease. Immunohistochemical analysis of diseased periodontal tissue demonstrated the cellular sources of IL-17E to be endothelial cells and infiltrating leukocytes. No expression of IL-17E was detected in oral keratinocytes. However, oral keratinocytes were demonstrated to express both IL-17RA and IL-17RB and therefore identified as a target for IL-17E signaling. Indeed, using an *in vitro P. gingivalis* biofilm model, IL-17E was found to negatively regulate the *P. gingivalis* induced expression of neutrophil pro-chemotatic chemokines; CXCL5 (ENA-78) and CXCL8 (IL-8).
Conclusions: IL-17E derived from endothelial cells and invading leukocytes play a role in the pathogenesis of periodontal disease. IL-17E has been demonstrated to be a negative regulator of oral

immunity. IL-17E can down regulate the expression of key neutrophil chemo-attractants and therefore possibly inhibit neutrophil chemotaxis into the periodontium. As neutrophils play a key role in the early events associated with periodontal disease progression, the data suggests IL-17E is a rational target for therapeutic intervention.

P0089
Impaired pulmonary immunity after cerebral ischemia

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Purpose/Objective: Severe cellular immunodepression is a frequent phenomenon in stroke patients, which is associated with a high rate of infectious complications and worse clinical outcome. Up to 20% of stroke patients develop pneumonia with worse recovery and high mortality. Clinical and experimental studies have shown that stroke induces a long-lasting suppression of cell-mediated immune responses in blood and lymphatic organs. The dynamics of quantitative and functional changes in lung immune cells has not been studied so far. Here we analysed, in a clinically relevant mouse model, the pulmonary immune response at different time points after stroke.
Materials and methods: In a well-established mouse stroke model (middle cerebral artery occlusion for 60 min) we characterized quantitative and functional changes of myeloid and lymphoid immune cell populations *ex vivo* in lung and spleen at different time points after stroke. The early pulmonary host response during bacterial pneumonia was analyzed in a model of aspiration-induced pneumonia. Phenotypic and functional analyses were done by flow cytometry, multiplex cytokine analysis of BAL fluid, plasma and cell culture supernatants after *ex vivo* TLR-ligand stimulation, and by assessing phagocytic activity.
Results: Analysis of cellularity showed decreased numbers of lymphocytes, dendritic cells and macrophages in lungs at different time points after stroke. Functional analyses indicated an impaired cytokine production in both lung cells and splenocytes after *ex vivo* TLR and T cell mitogen stimulation. First results of the *in vivo* host response to aspiration of low dose *Streptococcus pneumonia* that does not induce infection in naïve mice indicate an early and prolonged hyperinflammatory response with increased cytokine production in stroke mice.
Conclusions: Our data suggest that similar to the changes in lymphoid organs and peripheral blood also pulmonary immune cells show signs of impaired function after cerebral ischemia. The precise mechanisms of altered pulmonary immunity including the recruitment of peripheral immune cells during bacterial lung infection after acute CNS injury needs to be further investigated and may lead to new therapeutic strategies to prevent infections and improve outcome in stroke patients.

P0090
In vitro, induction of BAFF and APRIL expression by A549 and BEAS-2B airway epithelial cells

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Purpose/Objective: The production of BAFF and APRIL by epithelial cells contributes to local accumulation, activation, class switch recombination, and antibody production by B cells in the airways. Here we aimed to characterise BAFF and APRIL production by the cultured airway epithelial cells, A549 and BEAS-2B.

Materials and methods: §The total RNA was extracted and collected from A549 cells and Beas- 2b and RT-PCR was applied. Human BAFF ELISA and Il-8 were used to measure the protein.

§ *in vitro*, we investigated if cultured epithelial cellseither A549 or Beas-2b can be stimulated to produce BAFF or APRIL by viral infection, dsRNA or cytokines stimulation.

Results:

1 Induction of BAFF and APRIL mRNA in A549 cells after 12h stimulation
Induced expression of BAFF and APRIL mRNA in A549 cells varied according to the stimuli used. For example, BAFF mRNA was observed at 12hrs post INF β stimulation and IFN α and there was no expression for APRIl
2 Induction of BAFF mRNA in Beas-2b cells after 12h stimulation
Expression of mRNA BAFF has been detected post stimulation with IFN β and IFN α.
3 I INF $\boldsymbol{\beta}$ time course
Among the stimulantsfactors used, we have found that IFN §was significantly induced BAFF expression. Thus, weaimed to see in which time point.
4 RSV infection of cultured BEAS-2B cells induces BAFF expression by an interferon $\boldsymbol{\beta}$ dependantmechanism.

Conclusions: Collectively our results indicate that airway epithelial cells can produce BAFF in an interferon dependant manner. Suggesting that the Airway epithelial could help support B cell growth development and Ab production in the Lung.

P0091
Increased hyaluronic acid in the airway following influenza infection contributes to heightened susceptibility to secondary bacterial infection

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Purpose/Objective: Severe respiratory viral infections are often associated with bacterial complications. We have previously demonstrated the importance of the regulation of innate immunity in this altered susceptibility. This current work aims to elucidate the influence of airway hyaluronic acid (HA) on the innate immune rheostat following respiratory viral infection.

Materials and methods:

1 C57BL/6 mice were infected with 2–15 PFU/mouse A/PR8/34 (H1N1) influenza. HA content in bronchoalveolar lavage (BAL) fluid was determined by ELISA. Cell surface marker expression was determined by flow cytometry.
2 Lung integrity was altered by intranasal administration of MIP-2 or histamine.
3 Alveolar macrophages isolated from naïve mice were incubated with HA fragments for 4 h, allowed to recover for 18 h, then stimulated with LPS for 20 h. Cytokine levels in cell culture supernatants were determined by ELISA.
4 Mice were treated with 50 $\boldsymbol{\mu}$g HA 2 h prior to infection with 10^4 CFU/mouse D39 *Streptococcus pneumoniae*. Organ homogenates were cultured to determine the number of recoverable bacteria.

Results: HA content in the airway was increased by four log units following influenza infection, with a significant increase in the amount of HA persisting for more than 6 weeks. There was a substantial loss of integrity in the lung post viral infection, as determined by airway albumin content and staining of epithelial damage markers. Replicating this damage, however, by inducing infiltration of neutrophils or by disrupting endothelial tight junctions did not result in a substantial increase in HA.

CD44 is one of the major cellular receptors for HA on leukocytes and is highly expressed by alveolar macrophages (AM). CD44 has been previously shown to play a key role in the resolution of inflammation, and can trigger expression of the TLR negative regulator A20 when complexed with HA. Here we demonstrate that *ex vivo* exposure of AM to HA results in a significant desensitisation to LPS challenge. *In vivo*, introduction of HA into the airway of mice prior to bacterial challenge markedly reduced bacterial clearance and was associated with reduced cellular recruitment to the site of infection.
Conclusions: Following influenza-induced inflammation, increased hyaluronic acid in the airway contributes to the desensitisation of alveolar macrophages to TLR stimuli with a resultant increase in susceptibility to secondary bacterial infection.

P0092
Induction of pro-inflammatory cytokines in human NALT and peripheral blood by domain 4 pneumolysin

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Purpose/Objective: Pneumococcal infection causes significant morbidity and mortality worldwide. Currently available vaccines are either not effective in young children or limited in serotype coverage. Pneumolysin (Ply) is a protein toxin that is conserved in virtually all serotypes of pneumococci. Ply has four domains, which together induce pore formation on cell membranes. The fourth domain (D4Ply) is important in cell binding. Our previous studies have shown that D4Ply is a potent activated of T cell proliferation. We have studied the induction of cytokines in immune cells from both human nasal-associated lymphoid tissue (NALT) and peripheral blood.
Materials and methods: Mononuclear cells (MNC) from adenotonsillar tissues and peripheral blood mononuclear cells (PBMC) were isolated from children and adults undergoing adenotonsillectomy. $CD14^+$ monocyte and $CD3^+$ T lymphocytes were isolated from PBMC using MACS magnetic cell separation. Recombinant domain 4 Ply (D4Ply), which lacks toxicity was co-cultured with adenotonsillar MNC, purified monocytes or T cells. Expression of different cytokines including TH1, TH2 and TH17 cytokines was analysed by cytometric bead array, intracellular cytokine staining and ELISA. Activation of Toll-like receptor signaling was examined by RT-PCR array.
Results: D4Ply induced a predominant pro-inflammatory cytokine profile including IL-1β, Il-6, TNF-α (monocytes), IL-17 and INF-γ (T cells). D4Ply induced a stronger INF-γ response in PBMC, mainly by $CD8^+$ T cells, whereas there was a stronger IL-17 response in $CD4^+$ adenotonsillar MNC. The RT-PCR array showed that D4Ply stimulation of monocytes induced up-regulation of key genes in Toll-like receptor signaling pathway.
Conclusions: D4Ply activates both T cells and antigen presenting cells and can induced significant pro-inflammatory cytokine expression. Further studies are underway to examine the immunological pathway of cell activation by D4Ply and explore its potential as a human vaccine against pneumococcal infection.

P0093
Infiltration of ROR gamma t positive lineage negative lymphocytes into the lung during lipopolysaacharide-induced acute lung injury

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Purpose/Objective: The hallmark features of acute lung injury (ALI) include neutrophilic infiltration with the release of inflammatory cytokines causing damage to the alveolar epithelial-capillary endothelial barrier leading to pulmonary oedema. IL-17 produced by lymphocytes is known to enhance recruitment of neutrophils by promotion of other inflammatory cytokines, including IL-8 and IL-6. However, detailed characterisation of the lymphocytes infiltrating the lung in the early phase of ALI has not been reported.
Materials and methods: Lung tissue cells were characterised by flow cytometry in a murine model of ALI induced by intratracheal lipopolysaacharide (LPS).
Results: We observed increased numbers of lymphocytes in the lung 24 h after LPS treatment, including an increase in retinoic acid-related orphan receptor-gamma t $(ROR\gamma t)^+$ lymphocytes. $ROR\gamma t^+$ lung lymphocytes were observed to be predominantly $CD4^+$ T cells (18%), $CD8^+$ T cells (20%) cells and a Lin^- population (40%). Furthermore, we characterised the expression of a variety of cell surface markers associated with the innate lymphoid cells in the $ROR\gamma t^+Lin^-$ population in the lung and ex-vivo stimulation of cells was used to confirm IL-17 production. Using an IL-17KO mouse we have demonstrated a key role for this cytokine in the recruitment of neutrophils into the lung, as we observed a significant reduction in neutrophil numbers in the absence of IL-17.
Conclusions: These results identify a key role for innate cells, more specifically a $ROR\gamma t^+Lin^-$ population in the lung that contribute to IL-17 production and therefore inflammation in ALI.

P0094
Influence of metals onto sIgA, IgA1 and IgA2 production in saliva in patients undergoing implantation therapy in dentistry

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Purpose/Objective: Purpose of the study: The aim of this study was to compare sIgA, IgA1 and IgA2 production in saliva before, during and after implantation therapy in patients undergoing implantation therapy in dentistry.
Materials and methods: Material and methods: Saliva from 19 patients was collected before implantation therapy, from 15 patients after implatation and before prosthesis and from 16 patients after implantation and prothetic therapy. Secretory IgA, IgA1 and IgA2 antibody production was established using RID method.
Results: Results: The levels of all three types of IgA antibodies (secretory IgA, IgA1 and IgA2) in saliva of patients were increasing during implantation process, significantly increased levels after implantation and prosthetic therapy in comparison to levels before implantation therapy were found in secretory IgA and IgA2 antibodies.
Conclusions: Conclusion: Increased levels of type A antibodies during implantation therapy in saliva of patients show that defence immune reactions were already started.
Acknowledgement: The study was supported by research projects SVV 2012 nr. 264501 and PRVOUK-P28/LF1/6 ofMinistry of Education, Youth and Sports, Czech Republic and by project NT 13087-3 of the Internal Grant Agency, Ministry of Health, Czech Republic.

P0095

Intravaginal cell recruitment and upper genital tract pathology in mice pretreated with hyaluronic acid and infected with *Chlamydia muridarum*

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Purpose/Objective: Chlamydia is a widely spread chronic urogenital infection that essentially affects reproductive health worldwide. There is well acknowledged evidence that Chlamydia infection induces a range of immunopathological reactions, while the components of early immune response that might lead to protection from upper genital tract pathology and re-infection require further elucidation. In this study we evaluated early intravaginal cell recruitment in response to *Chlamydia muridarum* infection in mice pretreated with high molecular weight hyaluronic acid (HA) as a prospective delivery agent to assess how it effects both intravaginal cell recruitment and Chlamydia induced upper genital tract pathology.
Materials and methods: BALB/c mice were pretreated with 0.1% HA intravaginally (i.v.). Three weeks later they were infected i.v. with 10^6 IFU of *C. muridarum* and numbers and phenotypes of cells from vaginal washes were analyzed 48 h post infection (PI) by flow cytometry. Vaginal shedding and Chlamydia DNA in lower and upper genital tract were assessed at different time points post infection. Upper genital tract pathology (hydrosalpinx) was evaluated 4 weeks post infection.
Results: We found that HA induced a dramatic change in numbers and phenotypes of cells recruited to vagina in response to *C. muridarum* compared to untreated mice. Three fold increase in total cell numbers in HA treated mice was a accompanied with fivefold increase in $CD4^+$ T cells, while $CD8^+$ T cells predominated in vaginal washes of untreated mice. There was also an increase in neutrophils, macrophages and dendritic cells in HA-treated mice. We did not find a statistically significant difference in either vaginal shedding of bacteria or the levels of Chlamydia DNA in upper or lower tract between the groups, however a dramatic increase in incidence and severity of hydrosalpinx was observed in mice pretreated with HA. Possible correlations between the repertoire of cells recruited to the vagina in response to Chlamydia and Chlamydia induced genital pathology are currently under the study.
Conclusions: Our results demonstrate that introvaginal repertoire of cells recruited in response to Chlamydia can differ in mice with moderate or severe upper genital tract pathology even if they have the similar levels of pathogen DNA and vaginal shedding. Potential predictive value of this observation needs further elucidation.

P0096

Macrophage development in the neonatal mouse intestine

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Purpose/Objective: Macrophages (mϕ) are essential for both homeostasis and inflammation in the intestine. Adult gut mϕ are derived from pro-inflammatory monocytes, but acquire high phagocytic ability and production of IL10, allowing them to scavenge commensal bacteria without provoking inflammation. Aberrant mf reactions to microbiota drive inflammatory bowel disease (IBD), suggesting that the usual adaptation processes have been disrupted. The most important period for establishing the mucosal mϕ pool is likely to be during early life when the microbiota is becoming established. Here we have explored how intestinal mϕ acquire their normal pattern during the perinatal period.
Materials and methods: Intestine from CX3CR1-GFP reporter mice was analysed from E19.5 to adulthood using multi-parameter flow cytometry.
Results: Intestinal mϕ express very high levels of CX3CR1 before and around birth, but unlike adult mϕ, they are class II MHC^{neg} and are heterogeneous for expression of F4/80 and CD11b. One group is $F4/80^{hi}$ $CD11b^{int}$ and is similar to the yolk sac derived mf described in other tissues. The other subset is $F4/80^{int}$ $CD11b^{hi}$ and may be derived from foetal liver. These populations remain constant in number during the first 2 weeks of life, but $F4/80^{hi}$ $CD11b^{int}$ mf acquire class II MHC. Around weaning an influx of classical $Ly6C^{hi}$ monocytes gives rise to adult-type $CX3CR1^{hi}$ $F4/80^{hi}$ $CD11b^{hi}$ mϕ, presumably reflecting the increasing numbers of commensal bacteria.
Conclusions: Mϕ in neonatal intestine are a complex mix of cells of different origin, whose composition changes as the gut and its microbiota mature. These processes are likely to have important implications for future health and may contribute to susceptibility to IBD.

P0097

Methanoarchaea activate innate host defense mechanisms

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Purpose/Objective: The methanoarchaea Methanosphaera stadtmanae and Methanobrevibacter smithii are known to be part of the indigenous human gut microbiota. Although few studies focusing on the immunogenic effect of these strains are available, data for methanoarchaeal activation of innate host defense mechanisms are rare.
Materials and methods: We studied and report here on the cytokine response of peripheral blood mononuclear cells (PBMCs) and monocyte-derived DCs after incubation with *M. stadtmanae* and *M. smithii*.
Results: High cytokine release of TNF-a, IL-1-b, IL-8 and IL-6 was obtained after 20 h of stimulation with *M. stadtmanae*, whereas stimulation with *M. smithii* only lead to minor cytokine release. Moreover, phagocytosis of methanoarchaea by PBMCs and monocyte-derived dendritic cells (moDCs) was demonstrated by confocal microscopy and shown to be required for cellular activation by application of specific inhibitors. Western-Blot analysis demonstrated activation of MAP-kinases of stimulated moDCs after 90 min. Transfection of HEK293 cells with several common innate immune receptors did not reveal activation by the methanoarchaeal strains via TLRs or NLRs, thus pointing towards a different recognition mechanism.
Conclusions: This first comprehensive study of the inflammatory response of human immune cells to M. stadtmanae and M. smithii strongly suggests that methanoarchaea are specifically recognized by components of the human innate immune system and hence most likely have an effect on the immunomodulation within the human gut.

P0099

Myeloid activators of RORγ t- innate lymphoid cells

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Purpose/Objective: Inflammatory Bowel Diseaseisaheterogeneous group of inflammatory disordersarising from defects inmucosalpermeability coupled with unregulated, hyperresponsive effector cells. Theroleofadaptive immunity in IBD has been focusofmuch investigation, but the part played by innateimmunesystemremainspoorly defined. The innateimmune systemisanevolutionarily ancient componen to fmammalianimmunity and, as such, may provide important clues to

the aetiopathogenesis of IBD. It has previously been determined that an IFN-y producing, RORγt$^-$ subpopulation of innate lymphoid cells (ILC) is a potent inducer of innate colitis in mice. RORγt$^-$ ILC cells differentiate from RORγt$^+$ ILC as a result of largely unknown molecular signalling. We report here on the myeloid populations responsible for activating these colitogenic LTi-like cells and on the cytokines involved in the activation process.

Materials and methods: CD40-mediated colitis was induced in Rag2$^{-/-}$ mice on a C57BL/6 background. Mice were sacrificed 6 or 7 days post-injection. Lamina propria lymphocytes were analysed for expression of cell surface markers and intracellular cytokines using the MoFlo analysis, FACS analysis and real-time PCR.

Results: Anti-CD40 stimulation led to the induction of proinflammatory cytokines, with IL-12, IL-27 and IL-6 production peaking at 0.5 days postinjection (p.i.), TNF at 2 days p.i. andI L-23 at 7 days p.i. CD11b$^+$CD11c$^-$ (MHC II$^+$ Ly6C$^+$ F4/80$^+$ CD103$^+$ Ly6C/G$^+$) cells were the main producers of IL-12, IL-27 and IL-6. TNF was produced mainly by CD11b$^+$CD11c$^+$ (MHC II$^+$ Ly6C$^-$ F4/80^{lo} CD103$^+$ Ly6C/G$^-$) cells. CD11b$^+$CD11c$^+$ cells also secreted the greatest amount of IL-23 overall. IFN-γ production by RORγt$^-$ ILC correlated with the kinetic of IL-12, IL-27 and IL-6 production.

Conclusions: Activation of RORγt$^-$ ILC occurs rapidly upon CD40-crosslinking of myeloid cells and correlates to the onset of systemic wasting. CD11b$^+$CD11c$^-$ cells are the main myeloid population involved in the transition of ILC to a RORyt$^-$ phenotype. IL-6, IL-12 and IL-27 contribute to the induction of IFN-γ production by RORγt$^-$ ILC. The delayed production of TNF raises the possibility that activated RORyt$^-$ ILC subsequently stimulate myeloid cells. Although IL-23 is required for colitis development, the kinetic of IL-23p19 expression does not correlate with IFN-γ expression. Thus, IL-23 may instead be required to maintain IFN-γ expression at later stages.

P0100
Neutrophils are rapidly recruited by PT-gliadin itself

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Purpose/Objective: Gliadin, a component of gluten-containing cereals, triggers celiac disease (CD) in genetically predisposed individuals. We have shown that gliadin induces a profound increase in gut permeability. Histology of CD gut mucosa consistently shows massive influx of neutrophils. We aimed at studying the early immunological changes evoked by pepsin-trypsin digested-gliadin (PTG) to understand its role in neutrophil influx in CD.

Materials and methods: Ten C57BL/6 mice were given PTG or PBS by gavage. After 2h mice were sacrificed and duodenal tissue was examined for PTG effects on tight junction (TJ) integrity and immune cell subsets by fluorescence microscopy and flow cytometry. Neutrophil recruitment was monitored *in vivo* after luminal exposure to PTG or PBS for 2h by intravital microscopy using five Lys-GFP mice that have green-fluorescent neutrophils. Neutrophils were isolated from bone marrow of eight C57BL/6 mice, and chemotaxis to PTG, or fMLP as positive control, was monitored *in vitro* using a Taxi-scan assay.

Results: PTG caused redistribution of E-cadherin and ZO-1 TJ proteins, a measure of TJ disassembly, and increased numbers of GR1$^+$ granulocytes. Intravital microscopy revealed a rapid and massive neutrophil tissue influx within 2h after luminal challenge with PTG. This observation was confirmed in Taxi-scan assay.

Conclusions: PTG induces a massive neutrophil recruitment in the mouse intestinal mucosa. *In vivo* and *in vitro* models that allow real-time monitoring of neutrophils movements strongly suggest that PTG itself is a chemo-attractant factor for neutrophils.

P0102
Resident and pro-inflammatory macrophages in the colon represent alternative context dependent fates of the same Ly6Chi monocyte precursors

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Purpose/Objective: Macrophages (mϕ) are essential for intestinal homeostasis, but are also central to the pathology of inflammatory bowel disease. It is not clear whether distinct populations of mϕ are responsible for these different functions, or if the resident mϕ change during inflammation. We set out to investigate the phenotypic and functional characteristics of mϕ present in the steady state colon and during acute experimental colitis, and sought to determine the origin of these cells.

Materials and methods: CX3CR1-GFP reporter mice were used for phenotypic analysis of colonic mϕ. Acute colitis was induced by feeding 2% DSS in drinking water for up to 6 days. For adoptive transfer studies, Ly6C^{hi} BM monocytes from CX3CR1-GFP mice were transferred into unmanipulated CCR2$^{-/-}$ mice.

Results: We show here that most resident mϕ in resting mouse colon express very high levels of CX3CR1, are avidly phagocytic and MHCII^{hi}, but are desensitised to TLR stimulation and produce IL10 constitutively. A smaller population of CX3CR1^{int} cells is present in resting colon which is phenotypically heterogeneous, responds robustly to TLR ligation and expands dramatically during experimental colitis. Using a combination of gene profiling, immunophenotyping and adoptive transfer experiments, we demonstrate that rather than representing independent mϕ populations, CX3CR1^{int} and CX3CR1^{hi} cells in the steady state intestine represent cells at different stages of a progressive differentiation continuum. Under normal conditions, 'inflammatory' Ly6C^{hi} CCR2$^+$ monocytes enter the mucosa and differentiate into CX3CR1^{hi} mϕ through a CX3CR1^{int} intermediary stage. This phenotypically identifiable process is accompanied by a switch in cytokine production, TLR responsiveness and gene expression. Importantly, during inflammation this differentiation process is disrupted, leading to an accumulation of TLR-responsive TNFa-producing CX3CR1^{int} mϕ.

Conclusions: These results provide the first evidence that 'resident' and 'inflammatory' mϕ in the colon may be derived from the same monocyte precursor whose ultimate fate is determined locally and depends on the presence or absence of inflammation. Targeting these events could offer routes for therapeutic intervention in IBD.

P0103
Role of glucocorticoid receptor in regulation of pulmonary dendritic cells gene expression

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Purpose/Objective: Since the lung is constantly challenged with antigens, airway epithelial cells are important to control local immunity in order to avoid chronic inflammation. Recently we could demonstrate a role of epithelial cell derived prostaglandin E2 (PGE2)

in modulating IL12 and TNFalpha synthesis of respiratory tract dendritic cells (DC).
Materials and methods: In order to further investigate the involved pathways we analyzed the gene expression of murine bone marrow derived dendritic cells (BMDC) treated with airway epithelial cell conditioned medium (ECCM-DC) using microarray.
Results: Among the 97 genes being regulated by airway epithelial cells 61 were up- and 36 were down-regulated. The two most upregulated genes were Ms4a8a (18.914-fold) and Chi3l3 (18.513 fold). Both genes are known to be highly expressed in alternatively activated macrophages (M2). Surprisingly Ms4a8a and Chi3l3 gene expression was not affected by PGE2. When analyzing transcription factor binding site over representation in genes regulated by epithelial conditioning, clustering of sites for nuclear receptors was observed, e.g. glucocorticoid receptor (GR). Since glucocorticoids are known to be important for the induction of M2 macrophages we investigated the potential role of GR in modulating Ms4a8a and Chi3l3 expression in ECCM-DCs. Application of Dexamethasone to BMDCs induced the expression of Ms4a8a and Chi3l3 which was further potentiated by treatment with ECCM. In line with this, pharmacological inhibition of GR by RU486 as well as genetic deletion of GR could inhibit ECCM mediated induction of Ms4a8a and Chi3l3 in BMDCs.
Conclusions: Our results indicate a physiological role of glucocorticoids in regulation of pulmonary dendritic cell gene expression and function via GR.

P0104
TAM receptors and their role in the regulation of lung innate immunity

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Purpose/Objective: The role of TAM receptors are well characterised in autoimmunity, developmental biology and cancer biology. Their role in infectious diseases is now being elucidated. We have strong preliminary data showing that TAM receptors (Axl and MerTK in particular) become significantly up-regulated on alveolar macrophages following primary influenza infection, and this may account for the high rate of bacterial co-infection in hospitalised patients. In this study, the role of TAM receptors in various infection models was investigated and de-sensitisation of airway macrophages to bacterial infection following influenza infection was assessed.
Materials and methods: Using murine model, we extensively characterised TAM receptor expression profiles on various innate immune populations over 6 weeks post primary influenza infection. We have also characterised the expression of both Axl and MerTK following bacterial and fungal infections. We also manipulated Axl-GAS6 (Axl ligand) axis and monitored subsequent alteration in inflammatory cytokine production profile and phagocytic ability of alveolar macrophages.
Results: The expression profile of Axl on both alveolar macrophages and on neutrophils following influenza infection became significantly up-regulated, remaining higher than at its homeostatic level even 6 weeks after initial infection. Furthermore, Axl also becomes up-regulated during bacterial infection, and Axl expression shows 'Dose-response' to bacterial burden. Preliminary data also showed that the pre-treatment of alveolar macrophages with the Axl ligandGAS6 reduces production of inflammatory cytokines and TLR desensitisation.
Conclusions: This study has elucidated information into the behaviour of Axl and MerTK expression following various infection models. Sustained up-regulation of Axl following primary influenza infection has significant clinical implications as it is involved in the desensitisation of TLRs, which may cause patients to have a predisposition to secondary bacterial complications. Moreover, the manipulation of Axl-GAS6 axis has allowed us to gain insight into the phenotypic changes of alveolar macrophages brought about by influenza infection. The results of this study would allow us to develop a new approach in the intervention of secondary bacterial complications via the antagonism of Axl in the later stages of diseases.

P0105
The role of cathelicidins in protection against pathogenic lung infections

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Purpose/Objective: Cathelicidins are cationic host defence peptides with key roles in innate immunity. The sole human cathelicidin gene *CAMP* encodes the human antimicrobial protein of 18kDa (hCAP18), that is proteolytically cleaved to active peptides, with the predominant form being LL-37. LL-37 has been shown to perform a diverse array of microbicidal and immunomodulatory roles during infection. Mice deficient in the murine orthologue of *CAMP* (*Camp*) have increased susceptibility to bacterial infection in multiple systems, demonstrating the critical and non-redundant nature of cathelicidin-mediated protection in host defence against infection. However, the mechanisms underpinning this protection remain unclear, and the role of cathelicidins in host defence against viral infections has not been well characterised.
Materials and methods: Using *in vitro* and *in vivo* models of pulmonary infection with human respiratory pathogens *Pseudomonas aeruginosa*, respiratory syncytial virus (RSV) and influenza A virus (IAV), we demonstrate key antimicrobial and host immunomodulatory properties of cathelicidins.
Results: We report a number of different mechanisms by which cathelicidins promote pathogen clearance and protect against pulmonary infection, including: 1) direct anti-viral activity against both RSV and IAV, and induction of an antiviral state in airway epithelial cells, and 2) modulation of the cellular inflammatory response to infection; with diminished pathogen clearance and neutrophil influx in cathelicidin knockout animals (*Camp*$^{-/-}$) which could be both corrected in *Camp*$^{-/-}$ mice and enhanced in wild-type animals by therapeutic cathelicidin administration.
Conclusions: These data demonstrate key roles for cathelicidins in host defence against pathogenic bacterial and viral pulmonary infections and have the potential to inform the development of synthetic analogues as novel immunomodulatory antimicrobial therapeutics.

P0107
The role of dendritic cells in the initiation and maintenance in a pre-clinical model of ulcerative colitis

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Purpose/Objective: The mucus layer is an important component of an intact intestinal barrier and combined with appropriate immune regulation ensures that reactivity to luminal flora is kept at bay and intestinal tolerance is maintained. Evidence supports that a barrier breach results in an inappropriate immune response towards commensals inducing pathogenic T cells. In this study we analyze the role of dendritic cells in the initiation and maintenance of colitis in mice

lacking the protective mucus layer in the colon, thus the normal flora is in direct contact with intestinal cells, a situation that mimics the events triggering ulcerative colitis in humans.
Materials and methods: Single cells suspensions form $Muc2^{-/-}$ mice, lacking the gene for mucin 2 and thus lacking the protective mucus layer specifically in the colon are studied by multicolor flow cytometry as well as whole tissue lysates are analyzed by cytometric bead arrays.
Results: Single cell suspensions of colon LP were analyzed in regards to innate cell populations. These analyses revealed a significant increased of neutrophils in $Muc2^{-/-}$ mice. A 'cut off value' discriminating inflamed from non-inflamed $Muc2^{-/-}$ mice was established using the relative amount of neutrophils detected. According to this separation we further identified differences in dendritic cell subpopulations in the colon, revealing that the colon of inflamed animals had higher percentages of $CD11b^+$ DC, while at the same time the number of $CD103^+$ $CD11b^-$DC decreased though the relative amount of $CD103^+$ $CD11b^+$ DC did not alter in inflamed colon LP. Cytometric bead arrays of whole tissue lysates of parallel colon samples revealed a local increase in typical pro-inflammatory cytokines specifically a significant increase in IL-6, IL-17a and IFN-γ in later stages of the inflammation.
Conclusions: Together these findings suggest that the infiltration of neutrophils and monocytes initiate a cascade of events resulting in a Th17 biased immune response against luminal antigens.

P0108

The role of Th17 response towards a protein-based *Streptococcus pneumoniae* vaccine

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Purpose/Objective: Current vaccines to prevent *S.pneumoniae* infection are based on the induction of antibodies (Ab) against some of the more than 90 serotype-specific capsular polysaccharides. A different strategy to a successful vaccine could be based on broadly cross-protective protein antigens. Additionally, this strategy offers the possibility to exploit both arms of the immune system, coupling Ab-mediated to cell-mediated immunity. This is important as several findings suggest that natural immunity to *S.pneumoniae* might be acquired independently of anticapsular Ab and implicate IL17-producing $CD4^+$ T helper (Th17) cells. With our study we set to unveil the contribution of Th17 responses to the resolution of pneumococcal infection in mice.
Materials and methods: We assessed quantity and quality of T cell responses in mice immunized with different vaccine formulations and via different routes. Then, we adoptively transferred serum or CFSE-labeled $CD4^+$ T cells from vaccinated mice to naïve recipients prior to bacterial challenge. We closely followed infection kinetics and assessed weight loss/ survival of mice, bacterial counts in lung and blood, as well as recruitment of immune cells into the lung.
Results: Intranasal (i.n.) vaccination with a mucosal adjuvant induces higher Th17 responses than subcute (s.c.) administration with alum. Yet, s.c. induced responses can be enhanced by co-formulation with TLR agonists. Vaccine-induced CFSE-labeled $CD4^+$ T cells rapidly accumulate in the lung of infected mice and can contribute directly to protection. Accordingly, recruitment of other cell types is enhanced in actively or passively immunized mice, leading to lower bacterial counts and greater survival of mice.
Conclusions: The role of Th17 cells in pneumococcal infection was studied so far only after direct vaccination in different mouse knockout models. Applying adoptive transfer of vaccine induced $CD4^+$ T cells, we could unequivocally confirm their protective potential. This underlines the added value of a protein-vaccine that can induce both antibodies and T cells over a solely Ab-inducing glycoconjugate. Application of optimized vaccine formulations and administration routes for the induction of T cell and antibody responses should allow to exploit the combined potential of both arms of the immune system.

Poster Session: Innate-Like Lymphoid Cells

P0109

A novel model of MHC class I-related (MR1)-mediated activation of MAIT cells

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Purpose/Objective: Mucosal associated invariant T (MAIT) cells are an innate-like T cell populationthat comprise ~10% of the $CD8^+$ T cell compartment, express a semi-invariant (Vα7.2) T cell receptor and are restricted by the non-polymorphic, highly evolutionarily conserved MHC class I-related 1 (MR1) protein. While MAIT cells are activated by bacteria in an MR1-dependent manner the ligand(s) presented by MR1 and how it is processed are yet be defined. We sought to develop an *in vitro* model of MR1-dependent MAIT cell activation that would enable us to address these questions.
Materials and methods: Human PBMC or THP-1 cells were incubated overnight with *E. coli* and MAIT cell activation was assessed by intracellular cytokine staining and flow cytometry. The effect of blocking antibodies (MR1, IL-12, IL-18), pharmacological inhibitors, or siRNA-mediated gene knockdown was investigated. Co-localisation of MR1 with other cellular markers was assessed by confocal microscopy.
Results: Overnight stimulation of PBMC with *E. coli* lead to specific activation of MAIT cells, as assessed by CD69 and interferon-γ expression. This was partially inhibited by antibody blockade of MR1 but not IL-12 and IL-18. In contrast stimulation of purified $CD8^+$ T cells by *E. coli*-exposed THP-1 cells was entirely MR1-dependent within the first 6 h but by 20 h was mediated by both MR1 and IL-12 and IL-18. In both systems, inhibitors of endosomal acidification and of lysosomal cathepsins inhibited MAIT cell activation. Confocal microscopy revealed partial co-localisation of MR1 with lysosomes and surface expression of MR1 following exposure to *E. coli*. Furthermore, siRNA-mediated knockdown confirmed the requirement of β-2-microglobulin for efficient MR1-dependent activation of MAIT cells.
Conclusions: We describe a novel model MR-1-dependent activation of primary human MAIT cells. This model reveals that MR1-dependent activation is more rapid than cytokine-mediated activation and is dependent upon endosomal acidification, cathepsins, and β-2-microglobulin. This model will assist in further dissection of the MR1 pathway and identification of the microbial ligand(s) that it presents.

P0110

A search for non glycosphingolipid ligands as self lipids, causing CD1d-autoreactivity of type II Natural Killer T (NKT) lymphocytes

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Purpose/Objective: NKT cells make up a potent immunomodulating subset of T lymphocytes that is activated by self-lipids presented by CD1d molecules. So far, the identity of most self-lipids presented by CD1d underlying the autoreactivity of NKT cells are unknown. In this study, we set out to identify self-lipids causing autoreactivity of a sulfatide reactive, CD1d restricted, murine type II NKT cell hybridoma, XV19.
Materials and methods: To this end we fractionated lipids from a highly stimulatory antigen presenting cell (APC), A20CD1d TD and tested the stimulatory capacity of the type II NKT cell hybridoma, XV19.
Results: Two active glycoshpingolipids (GSL); glucosylceramide (Glcβ1-1′Cer) and galactosylcermide (Galβ1-1′Cer) were identified as active ligands for XV19 cells. The most potent isoform of the GSL was the lysoforms, lacking the fatty acid chain, followed by isoforms with the long fatty acid chain of C24. Thus, one NKT cell hybridoma could recognize sulfatide, GlcCer and GalCer both as presented by APC in a CD1d-dependent manner, and when presented on plate bound CD1d. Furthermore, we show that XV19 cells were not dependent on GSL for natural CD1d autoreactivity, using APC deficient in all GSL.
Conclusions: These results suggesting that self ligands that determine CD1d-autoreactivity of XV19 cells are non GSL. Preliminary data suggest reactivity to non GSL, and investigations are ongoing to determine the role of non GSL in CD1d autoreactivity. Identifying endogenous lipid ligands that activate type II NKT cells will be of fundamental importance for our understanding of NKT cell activation in autoimmunity and tumor immunity.

P0111

Decreased IL-17$^+$ CD16$^+$ and IL-10$^+$ CD16$^+$ NK cells in Behçet's disease

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Purpose/Objective: Behcet's disease (BD) is a multi-system inflammatory disorder with recurrent inflammation attacks. BD pathogenesis includes genetical, environmental and immunological factors. The main genetic susceptibility is HLA-B*51, but the mechanism is unknown. HLA molecules play a key role with the interaction with natural killer (NK) cells. NK cells are also critical components of the innate immune response by virtue of their capacity to produce a variety of cytokines and have to ability to both lyse target cells and serve as regulators of immune responses by releasing Th1, Th2 and Th17 type cytokines. In this study, cytokine secretion and cytotoxic activity of NK cells in BD were investigated.
Materials and methods: The study group consists of BD patients (n = 26, mean age = 37.2 ± 9.7) with mucocutaneous involvement and healthy subjects (n = 12, mean age = 32.5 ± 8). The patients have not received any immunsupresive treatment. Peripheral blood mononuclear cells (PBMC) were used as effector cells and K562 cell line was used as target cell. PBMCs stained with CD107a were incubated with K562 cells with an effector: target (10:1) for 4 h. Cells were stained with anti-CD16 antibody for the determination of CD107a expression by flow cytometry. Cytotoxic activity (by using CFSE-labeled K562 as target cells), expression of NK cell receptors and surface markers, intracellular IL-5, IL-10, IL-17 and IFN-γ levels in $CD16^+$ NK cells were also determined by flow cytometry. Statistical analyses were performed by Mann Whitney U test.
Results: The percentages of NK, $CD16^{bright}CD56^{dim}$, $CD16^{dim}CD56^{bright}$ subsets, NKp46 and NKG2D expressions were found similar in both groups. However there was a significant difference in cytokine secretion.Although IFN-γ secreting NK1 cell subsets were found to be higher(P = 0.001), IL-5$^+$ CD16$^+$ NK2, IL-17$^+$ CD16$^+$ NK17 and IL-10$^+$ CD16$^+$ NKregulatory cells significantly decreased in BD patients. (P = 0.001). Even CD107a expression was significantly decreased in BD, cytotoxic activity did not show any difference between groups with CFSE (P = 0.03).

Conclusions: Our findings revealed that increased NK1 cell subsets and also decreased numbers of NK17 and NK regulator cell subsets might play role in BD pathogenesis.

P0112

Effect of T follicular helper cells on regulation of mucosal immunity to influenza haemagglutinin by novel immunological adjuvants

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Purpose/Objective: Stimulation of the innate immune system is known to have an important role in the initiation and regulation of adaptive immunity. Therefore, inclusion of some immunological adjuvants such as Toll-like receptor ligands, which trigger early innate responses to enhance the adaptive responses, is crucial to vaccine effectiveness. T follicular helper cells (T_{FH}) have recently been shown to be crucial in germinal centre function and in regulation of adaptive immunity. The aim of the study is to investigate whether and how TLR ligands regulate T and B cell immunity to some respiratory tract pathogens through T_{FH} cells.
Materials and methods: Peripheral blood, nasopharyngeal swab and adenotonsillar tissues were collected form children and adults undergoing adenotonsillectomy. B cell antibody production analysed ELISA. T_{FH} cells and effect of TLR ligands on their function were analysed by flowcytometry and intracellular cytokine staining. Kinetics of antibody and cytokine production will also be analysed by ELISA.
Results: The proportion of T_{FH} cells in adenotonsillar tissue was significantly higher in children than that in adults. Stimulation of adenotonsillar cells by CpG-DNA was shown to increase the numbers of T_{FH} cells and this was consistent with the finding that CpG-DNA significantly enhance the antibody production to haemoglutinin (HA) of seasonal influenza virus H1N1 (sH1N1) antigen in adenotonsillar cells. However, TLR-2 ligand (BLP) seems to downregulate both the proportion of T_{FH} cells and the antibody level to sH1N1.
Conclusions: CpG-DNA promotes T_{FH} cells in nasal-associated lymphoid tissue which is correlated with the enhancement of influenza HA-specific antibody production. Understanding the mechanisms by which TLR ligands regulate adaptive immunity through T_{FH} cells may lead to novel vaccines against respiratory infections.

P0113

IL-33 induces innate lymphoid cell-dependent airway inflammation by activating mammalian target of rapamycin

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Purpose/Objective: Recently, novel type 2 innate lymphoid cells (ILCs) have to been shown to be important mediators of anti-parasitic immunity in the gut and allergic inflammation in the airways. The IL-1 family cytokine IL-33 is a potent activator of ILC function yet the mechanisms by which this cytokine modulates their activation are not clear. The purpose of this study was to determine the role of mammalian target of rapamycin(mTOR) signalling pathways in the activation of ILC responses and the induction of airway inflammation by IL-33.
Materials and methods: We determined the effect of IL-33 on mTOR activation and examined the impact of the mTOR pathway *in vivo* using a model of IL-33-induced lung inflammation.
Results: IL-33 induced mTOR activation via p110d phosphoinositide 3-kinase in ST2-(IL-33 receptor)-positive Th2 cells and ILCs. Furthermore, treatment with the mTOR inhibitor rapamycin reduced IL-33-induced IL-5 and IL-13 production by both T_H2 cells and ILCs *in vitro* and *in vivo*. Administration of IL-33 to wild-type (WT) mice induced airway inflammation whilst *ex vivo* analysis analysis of lung digests identified ILCs as the predominant IL-5 and IL-13-producing cell type. Furthermore, adoptive transfer of WT ILCs to ST2-deficient mice recapitulated IL-33-driven airway inflammation. Importantly, co-administration of rapamycin reduced IL-33-dependent ILC-mediated inflammation, cytokine secretion and mucus deposition in the airways.
Conclusions: These data reveal a previously unknown and critical role for mTOR in IL-33-driven, ILC-dependent inflammation. Furthermore, our results suggest the possibility that manipulation of this signalling pathway might represent a target for therapeutic intervention of airway inflammation.

P0114

Innate lymphoid cells in models of IBD

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Purpose/Objective: The increasing prevalence of inflammatory bowel diseases (IBD) in the developed world reinforces the need to understand the mechanisms that lead to onset of inflammation in the gut. Previous work from our lab identified a population of RORgt-dependent, IL-23-responsive innate lymphoid cells (ILCs). These could drive inflammation in innate models of IBD. Subsequent IBD studies identified equivalent populations in humans. ILCs may therefore represent a novel target for therapy in some IBD subtypes, and understanding survival and functional aspects may be useful in this regard. We have investigated further differences between innate models of colitis, focusing on the role of and differences between ILC populations.
Materials and methods: We used the *Helicobacter hepaticus*-induced and αCD40-induced mouse models of innate colitis. Both models share requirements for IL-23, RORg and ILCs. However these innate models also differ as the former is a bacterially driven, chronic colitis and typhlitis, and the latter is bacterially independent, acute, and is primarily restricted to the proximal colon. We investigated the ILC populations in the absence of cytokines including IL-7 in addition to measuring the colitis.
Results: We show a difference between our models in the requirement for IL-7, a typical homeostatic cytokine, to promote colitis, as the αCD40 model was not IL-7-dependent. Similar to lymphoid tissue inducer cells, other ILCs also express the IL-7R. However, we show that signaling through IL-7 is not necessary for the presence of all ILC populations *in vivo*. Furthermore, in a model where IL-7 is important to promote disease, this is not due to a requirement of the cytokine for ILC development. IL-7 can modulate the pro-inflammatory cytokine milieu associated with disease.
Conclusions: We have used the differences between animal models of colitis to try to understand the relative importance of ILC populations and cytokines such as IL-7 in the development of colitis. The gut is a major source of IL-7, and we have shown that IL-7 is not just a

homeostatic cytokine but can also influence the inflammatory environment during disease.

P0115

Lymphocytes but not Rorgamma$^+$ cells are the source of LTb in ectopic lymphoid structures in inflammatory conditions

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Purpose/Objective: Lymphoneogensis is critically dependent on the interaction between LTβR expressed on mesenchymal cells and LTβ produced by RORg$^+$ LTi. Presence of tertiary lymphoid structures (TLS) that resemble lymph nodes in organization and local production of lymphoid chemokines (CKs) and cytokines have been reported in various chronic inflammatory diseases. Emerging evidence highlight the role of various stimuli and cytokines in supporting TLS formation, however it is not clear the role that LTβ/LTbR interaction plays in this process and it is still debated the role od adult LTi in driving TLS during inflammation. We used a model of ectopic lymphoneogenesis, in adenoviral infected murine salivary glands to dissect the role of LTβ/LTβR axis and identify the source of LTβ in the inflammatory aggregates.
Materials and methods: Quantitative RT-PCR and immunofluorescence was performed at different time points post viral cannulation (pc) in WT and knockout mice (LTβRKO, RAGKO, RORgKO, CD3eKO, IgHKO) in order to investigate the role of these molecules in TLS development.
Results: WT animals are characterized by the formation of TLS in the salivary glands with T/B cell segregation and FDC (follicular dendritic cell) network formation which recapitulates the events happening in lymphoid organogenesis including expression of LTβ and CKs (CXCL13, CCL21 and CCL19). LTβRKO displayed disorganisation of the inflammatory aggregates, lack of FDC and significantly decreased expression of the lymphoid CKs both at protein and mRNA level. Surprisingly, absence of LTi (RORgKO mice) did not significantly affect CKs production and aggregates organization. Conversely, severe impairment in the expression of lymphoid CKS and LTβ was observed in RAGKO mice, accordingly both T and B knockout showed a similar (though milder) phenotype to the RAG, suggesting that both T and B cells express LTβ and synergistically contribute to the production of this cytokine.
Conclusions: This data demonstrated that full maturation and maintenance of TLS is dependent on LTβR/LTβ axis and that the source of LTβ in the inflamed tissue, differently from the embryonic secondary lymphoid organs, is not provided by RORg$^+$ LTi but by infiltrating T and B lymphocytes.

P0116

Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles

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Purpose/Objective: Intestinal lymphoid follicles such as cryptopatches and isolated lymphoid follicles (ILFs) are lymphoid organs that develop postnatally under the influence of the intestinal microflora and other as of yet unidentified cues. The cells forming cryptopatches are RORγt (retinoic acid orphan receptor γ t)-expressing innate lymphoid cells (ILCs) believed to have lymphoid tissue inducing (LTi) function. Furthermore, RORγt$^+$ ILCs are an important source of interleukin-22, a cytokine essential for the defense against bacterial pathogens. In this study, I investigated the role of the aryl hydrocarbon receptor (AhR) for development and function of RORγt$^+$ ILCs.
Materials and methods: *Ahr*-deficient mice and mice fed with diet lacking natural AhR ligands were used to study the role of AhR for the development and function of RORγt$^+$ ILC. The effect of deficient AhR signaling in RORγt$^+$ ILC for the intestinal homeostasis was studied by using *Citrobacter rodentium* infection, a mouse model of attaching and effacing infections such as those with enterohemorrhagic *E. coli* strains.
Results: Mice lacking AhR had substantially reduced numbers of RORγt$^+$ ILCs and failed to develop cryptopatches and ILFs. In contrast to *Ahr*-proficient mice, RORγt$^+$ ILCs from mice lacking the AhR had very low levels of Kit expression. Kit is known to promote cell survival and proliferation in various cell types and Kit was recently reported to be required for the development and/or maintenance of intestinal RORγt$^+$ ILCs. Interestingly, dietary AhR ligands were found to control the pool size of RORγt$^+$ ILCs by maintaining the high Kit expression. In addition, consistent with the failure to expand the IL-22 producing RORγt$^+$ ILCs, *Ahr*-deficient mice were highly susceptible to *Citrobacter rodentium* infection.
Conclusions: Collectively, the data shows that AhR signals are indispensable for the expansion and/or maintenance of RORγt$^+$ ILC with LTi function required for the postnatal formation of intestinal lymphoid follicles.

P0118

TLR2 mediated propionibacterium acnes adjuvant effect on B-1 cell and on its phagocyte differentiation

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Purpose/Objective: B1 lymphocytes, like macrophages, are predominant in the peritoneal cavity. These cells are responsible for natural IgM and they are involved in innate immunity. They simultaneously express macrophages (CD11b/CD18) and lymphocytes (IgM, CD19, CD5) markers and are classified according to surface molecules, as B1a ($IgM^+/CD19^+$ $/CD11b^+/CD5^+$), B1b ($IgM^+/CD19^+$ $/CD11b^+$). Enriched B1b cell cultures can be obtained from mouse peritoneal adherent cells. When re-cultivated, they differentiate into a phagocyte derived from B1b (B1CDP) that expresses F4/80, CD11b and CD19 on membrane and also shows phagocytic activity. Recently, we demonstrated *in vivo* and *in vitro* the ability of heat-killed *Propionibacterium acnes* suspension and their soluble polysaccharide (PS) to increase B1b subtype and B1CDP on mice peritoneal cavity. Bacteria also induced *in vitro* and *in vivo* early phagocyte differentiation. The aim of this study was to verify if *P. acnes* effects are mediated by TLR2 and TLR4 on B1 and B1CDP.

Materials and methods: C57BL/6 wild type, $TLR2^{-/-}$ and $TLR4^{-/-}$ received a single injection of *P. acnes*, PS or saline. After 24 h, the cells from peritoneal exudatewere analyzed for B1 subtypes and B1CDP by flow cytometry. *Saccharomyces cereviseae* was labeled with CFSE to evaluate phagocytosis Internalized yeast by B1 or B1CDP was detected by flow cytometry.

Results: Bacteria decreased B1 $TLR2^+$ and it did not modify B1 $TLR4^+$ absolute number. *P. acnes* also elevated $TLR4^+$ absolute number and TLR2 expression/cell in B1CDP. PS increased only B1b $TLR2^+$. We demonstrated that the increasing in B1b and the phagocyte differentiation was mediated by TLR2, after bacterial stimulus, once in $TLR2^{-/-}$ mice we did not detected this effect.

The analysis of the phagocytic function in $TLR2^{-/-}$ and $TLR4^{-/-}$ mice revealed increasing B1b absolute number with internalized yeasts in *P. acnes* group; in contrast, these effect was observed in B1a subtype in the wild type group. Bacteria stimulus decreased B1CDP and also their phagocytic function on $TLR2^{-/-}$ group. Any effect was observed in PS treated groups.

Conclusions: The adjuvant effects induced by *P. acnes* on B1 cells, mediated undoubtly by TLR2, such as: increasing absolute number, differentiation into phagocytes and phagocytic activity, re-inforce the importance of this adjuvant and also the role of these cells on innate immune response.

Poster Session: Micro RNA

P0119

Functional role of microRNAs in the regulation of alternative activation in macrophages

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Purpose/Objective: Macrophages play a key role in maintaining the balance and efficiency of the immune response. T_H2 cytokines IL-4 and IL-13 trigger a state of alternative activation that is involved in the control of extracellular parasites such as helminths. Alternatively activated macrophages (AAMΦ) have also been implicated in tissue repair. However, inflammatory disorders like asthma and fibrosis are a result of a T_H2 weighted imbalance. Hence, macrophage responses must be tightly regulated. MicroRNAs, a short (~22nt) class of non-coding RNA, are one such immunomodulatory feedback mechanism that can regulate gene expression by targeting the 3' UTR of mRNA resulting in destabilization of the mRNA and/or inhibition of translation. We believe that with their ability for vast gene regulation, microRNAs could play a crucial role in the regulation of AAMΦ's by targeting genes and pathways critical for their induction and/or maintenance, thereby opening up new avenues for therapeutic intervention.

Materials and methods: To investigate the functional role of microRNAs in alternative activation, we have been using a murine model of filarial infection, *Litomosoides sigmodontis*, in which the nematode migrates to the pleural cavity and induces a T_H2 type response.

Results: Following parasite entry, the resident pleural cavity F4/80$^+$ macrophages proliferate and express the alternative activation markers, Arginase1, Relm- α and Ym-1. We have previously identified microRNAs associated with AAMΦ and in this study followed their expression in F4/80$^+$ macrophages over the course of *L. sigmodontis* infection, relating microRNA expression to alternative activation markers and cell division. We have also been evaluating alternative activation and miRNA expression profiles in IL-4-treated RAW 264.7 versus J774.1 MΦ cell lines.

Conclusions: We aim to establish these cell lines as lentiviral transduction targets for cross-linking and analysis of cDNA (CRAC), a technique that will provide in depth information on miRNA-mRNA interactions in AAMΦ.

P0120

Localized and generalized granulomatosis with polyangiitis nasal tissue displays specific disease associated micro-RNA profiles

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Purpose/Objective: To characterize miRNA profiles associated with localized and generalized GPA and to identify new miRNA targets of pathophysiological relevance. Disease specific alteration of miRNA expression has been found in chronic inflammatory diseases such as RA, Sjögren's, SLE or chronic inflammatory bowel disease. Yet, no investigations in vasculitides have been described.

Materials and methods: Nasal tissue samples of 14 patients with generalized and six patients with localized GPA, 10 disease controls (CRS: chronic rhinosinusitis with polyps) and 10 healthy controls (HC) were genome-wide screened (Affymetrix® miRNA-microarray chip 1.0). Differential expression was calculated by Mann-Whitney U-test; fold changes were based on the ratios of the medians of each experimental group. Results were visualized using TIBCO Spotfire®. Putative miRNA targets were predicted by combining three different computational methods: (i) PubMed search, (ii) Tarbase 5.0 (Diana labs), an algorithm for experimentally validated targets and (iii) microRNA Data Integration Portal (mirDIP).

Results: 1. From 847 human miRNAs screened, 24 displayed a GPA-specific expression pattern at cut-off level of a twofold change. For localized GPA 11 miRNAs were differentially regulated compared to generalized GPA. Further 7 miRNAs were unspecifically changed in GPA as well as in CRS.

2. Target prediction revealed previously experimentally validated targets involved in proliferation and differentiation of T- and B-cells, dendritic cells, fibroblast and of osteoblasts as well as apoptosis, hypoxia, angiogenesis and cellular adhesion. These are known pathways implicated in the pathogenesis of GPA. Potential targets also include proteinase-3, the specific GPA autoantigen, and lysosomal membrane protein-2, only recently described as an alternative autoantigen in GPA.

Conclusions: For the first time, this explorative survey compares disease associated miRNA profiles in nasal tissue samples of localized and generalized GPA patients. Predicted GPA specific miRNA targets belong to known pathological pathways of GPA. Considering the promising diagnostic, prognostic and therapeutical applications of miRNAs in other diseases, validation of the clinical relevance of our data will help to understand GPA on a molecular level as a key element in developing new therapies.

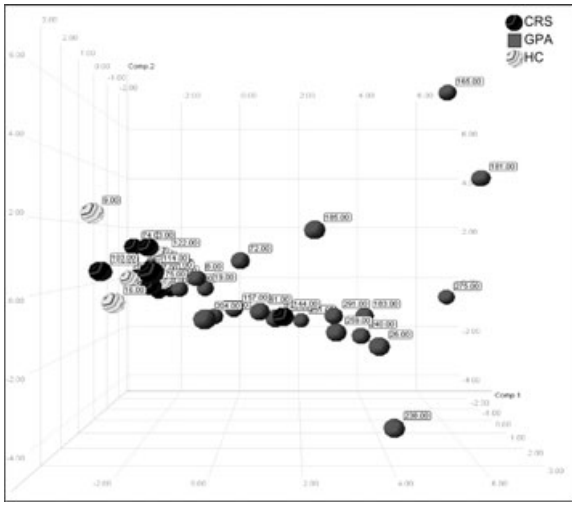

Figure 1. Principal component analysis: GPA patients form a different group from HC and CRS patients.

P0121

miR-142-3p, miR-185 and miR-181a, b, c and d control levels of the GARP protein, which in turn regulates processing and secretion of latent TGF-b1 in human T lymphocytes

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Purpose/Objective: How regulatory T lymphocytes (Tregs) suppress other immune cells is not completely understood. We previously proposed that human Tregs suppress at least in part through transformation of latent TGF-§1 into the active, immunosuppressive

cytokine. TGF-§1 activation by Tregs is confined to the cell surface, possibly by means of GARP, a transmembrane protein that binds latent TGF-§1 on Tregs. What controls GARP expression in T lymphocytes and the precise roles it plays in the TGF-§1 processing pathway are currently not known. In a collection of stable human T cell clones, we detected the GARP protein in stimulated Tregs, but not in T helper (Th) clones, some of which nevertheless expressed substantial amounts of the *GARP* mRNA. This suggests the existence of a post-transcriptional control of *GARP* expression in human T lymphocytes. Here, we set out to examine whether microRNAs (miRs) contribute to such a regulation.

Materials and methods: Using publicly available bioinformatics programs, we identified miRs predicted to target the 3′UTR of the *GARP* mRNA. Candidate miRs were tested by co-transfection with a reporter plasmid in 293T cells. Levels of miR expression in stable human T cell clones were quantified by RT-qPCR. The impact of miRs overexpression or inhibition was assessed in human T cells transfected with mimics or antagomirs, respectively.

Results: We identified 6 miRs, namely *miR-142-3p*, *miR-185* and *miR-181a*, *b*, *c* and *d*, that directly target the *GARP* 3′UTR in 293T cells, decrease endogenous GARP protein levels when transfected in Tregs and are expressed at higher levels in human Th clones by comparison to Tregs. Inhibition of *miR-142-3p* and *miR-185* in Th cells induced GARP protein expression. In Th cells transduced with GARP-encoding viruses, presence of the 3′UTR region targeted by the 6 miRs decreased GARP protein levels. Importantly, this correlated with reduced cleavage of pro-TGF-§1 and reduced secretion of latent TGF-§1.

Conclusions: We identified a miR-dependent mechanism of post-transcriptional control of GARP levels, which could play a role in regulating the amounts of latent TGF-§1 available for activation at the surface of human Tregs.

P0122

miR-182 and miR-10a are key regulators of treg specialisation and function during Th2 and Th1-associated inflammation

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Purpose/Objective: $CD4^+$ $Foxp3^+$ regulatory T cells are critical components of the immune system, required to control immune responses. The molecular identity of $CD4^+$ $Foxp3^+$ cells has been assumed to be largely independent of the immune environment.However, the recent identification of genes differentially expressed among $CD4^+$ $Foxp3^+$ cells is suggestive of functional heterogeneity.

Materials and methods: To investigate comprehensively whether $CD4^+$ $Foxp3^+$ cells are tuned to different responses and to identify regulatory pathways involved, $CD4^+$ $Foxp3^{gfp+}$ cells were isolated from Th1-rich or Th2-rich environments following *Leishmania major* or *Schistosoma mansoni* infection. Whole genome expression profiling and next generation sequencing revealed unique and dissimilar mRNA and miRNA profiles in $CD4^+$ $Foxp3^+$ cells from the different environments.

Results: *In silico* analyses, with extensive molecular validation demonstrated that distinct miRNAs are involved in determining the gene expression profile. MiR-10a was the strongest candidate 'master regulator' of $CD4^+$ $Foxp3^+$ cells in type-1 environments, whereas miR-182 was the most critical in type-2 environments. *In vitro* and *in vivo* systems further demonstrate that tight regulation of miR-182 and miR-10a is required for $CD4^+$ $Foxp3^+$ function.

Conclusions: These data support the notion that $CD4^+$ $Foxp3^+$ cells are specialized for different immune responses and identifies novel upstream miRNA pathways which influence Treg specialisation and function.

P0123

MiR-30* family negatively regulates BAFF synthesis in rheumatoid synoviocytes

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Purpose/Objective: In rheumatoid arthritis (RA) resident cells of the joint, fibroblast-like synoviocytes (RA FLS) acquire an aggressive phenotype in response to extrinsic factors such as PAMPs or DAMPs and intrinsic factors such as microRNAs. They produce large amounts of cytokines and among them the B cell-activating factor (BAFF) which allows them to collaborate with auto-immune B cells. We found that the miR-30* family of microRNAs (miR-30a*, d* and e*) was predicted to potentially target the 3'-UTR region of BAFF. As BAFF is also up-regulated in the skin and the serum of systemic sclerosis patients, the aim of this study was to evaluate the role of miR-30* in the regulation of BAFF synthesis in fibroblasts isolated from either RA or SSc patients.

Materials and methods: FLS and HDF were isolated from RA synovial tissues or from skin from SSc patients and were stimulated with TLR3 ligand (poly I:C) and IFN-γ for 3 days. RT-qPCR was performed to evaluate miRNA and mRNA expression. Transient transfection of RA-FLS and SSc-HDF with mimic miR-30* was performed using the Human Dermal Fibroblast NucleofectorTM kit from Amaxa. All assays were performed 24h post transfection.

Results: We first showed by qRT-PCR that like RA-FLS, SSc-HDF synthesize and released BAFF in response to poly I:C and IFN-γ. Conversely, HDF from normal subjects released BAFF only in response to IFN-γ. Using qRT-PCR, we demonstrated that miR-30a* and miR-30e* expression was strongly down regulated in RA-FLS, SScHDF and NHDH stimulated with either poly I:C or IFN-γ. Interestingly, NHDF which did not release BAFF in response to poly I:C expressed higher levels of miR-30a* and miR-30e* in response to poly I:C. MiR-30d* was not expressed constitutively or after activation by poly I:C and IFN-γ by each cell type. To evaluate whether miR-30* regulates BAFF expression in poly I:C and IFN-γ-activated RA-FLS and HDF, we transfected cells with miR-30* mimics. Transfection of the mimics induced a strong down-regulation of BAFF synthesis and release in response to poly I:C and IFN-γ. Using a luciferase test, we showed that miR-30* regulated directly BAFF mRNA 3'-UTR.

Conclusions: Our data strongly suggest a critical role of miR-30* in the regulation of the expression of BAFF which could play an important role in the regulation of the auto-immune response in RA and SSc.

P0124

Regulated expression of microRNA-21 is involved in the memory T-cell survival

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Purpose/Objective: MicroRNAs (miRNAs) are important post-transcriptional gene regulators required for proper development and function of immune cells. Although the dynamic expression of hematopoietic-specific miRNAs during consecutive phases of lymphocyte development in general is well documented, the specific role of single miRNAs in T cell activation and differentiation is still not well known. In this study we investigated the function of miR-21 in the process of T cell activation, and we report that miR-21 is a pro-survival factor in the context of memory T cell function.
Materials and methods: Using qRT-PCR we analyzed miR-21 expression in course of anti-CD3/CD28 stimulation of naïve ($CD4^+$ CD45RO-) and memory ($CD4^+$ $CD45RO^+$)-FACS sorted primary T cells, as well as in the Jurkat cell line. Viral vector harboring miR-21 inhibitor or scrambled-miR inhibitor sequence served to stably inhibit endogenous miR-21 in primary naïve and memory T cells as well as in Jurkat cells. Apoptosis rate was assessed by measuring mitochondrial potential. Immunoprecipitation of RISC complexes using mouse-anti-human-Ago2 monoclonal antibody followed by gene expression analysis was used to experimentally uncover T-cell specific miR-21 target genes.
Results: Resting memory $CD4^+$ T cells expressed five times more miR-21 than their naïve counterparts, suggesting involvement in the maintenance of the memory phenotype. Moreover, miR-21 was strongly upregulated upon TcR engagement in both primary and Jurkat cells, implicating it's contribution to the activation process. MiR-21 inhibition resulted in an increased rate of spontaneous apoptosis in *in vitro* activated T cell cultures, especially in the memory T cell population and induced massive spontaneous apoptosis in Jurkat cells. Immunoprecipitation of RISC complexes in Jurkat cells depleted of miR-21, followed by high throughput gene expression analysis allowed us to identify several potential miR-21 target genes relevant to T cell biology.
Conclusions: In conclusion, our data provide evidence that miR-21 is a key component in the process of T-cell activation by regulating T-cell survival signaling, especially in the memory T-cell population.

P0125

Regulation of microRNA 223 expression in a gouty arthritis

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Purpose/Objective: Introduction. Gout is an inflammatory chronic disease caused by deposition of uric acid crystals in the joint and connective tissues causing pain and disability. Current data suggest that gout is mediated by IL-1b that is produced due to the activation of the inflammasome pathway by uric acid crystals.In addition, neutrophil influx in the joint is the key initiator of a gout flare. miR-223 has been identified as a masterswitch molecule limiting neutrophil activation. In addition, we showed previously that this miR negatively regulates NLRP3 (an inflammasome component) and IL-1b production in human macrophages.

Objective. To investigate miR-223 expression and regulation in monocytes and neutrophils of gout patients.

Materials and methods: Methods. $CD14^+$ cells and neutrophils were isolated from gout patients ($n = 10$) and healthy donors ($n = 6$) peripheral blood using CD14 microbeads and polylymphoprep gradient buffer, respectively. $CD14^+$ from healthy donors were stimulated with LPS (10 ng/ml) IL-1beta (100−10 ng/ml), IL-6 (100 ng/ml), TNF alpha (10−100 ng/ml) or monosodium urate crystals MSU (1 mg−1 ug/ml) for different time points (24−72 h). Cells were harvested and miRNA extracted. Expression of miR-223 and endogenous control snRNA U1 was assessed by qPCR.
Results: Results. miR-223 expression in peripheral blood monocytes of patients withchronic gout was lower compared to healthy controls.This suggest that overproduction of IL-1b in chronic disease might be partially mediated by low levels of miR-223. *In vitro* studies revealed that MSU, IL-1b, TNFa and IL-6 significantly inhibited miR-223 expression in monocytes in all time points (24−72 h). In contrast, IL-10 strongly increased miR-223 expression. Interestingly, the levels of miR-223 in peripheral blood neutrophils were higher in gout patients compared to healthy controls.
Conclusions: A decrease in miR-223 expression in monocytes of chronic gout patients may contribute to uric acid crystals induced inflammasome activation and chronicity of disease. Upregulation of miR-223 expression in gout neutrophils may reflect the activation of mechanisms that limits neutrophils activation and lead to the resolution of gout flares.

P0126

Role of microRNAs in the differentiation of pro-inflamatory γδ T-cell subsets

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Purpose/Objective: The cytokines interferon-gamma (IFN-γ) and interleukin-17 (IL-17) are critical mediators of the pro-inflammatory activity of T-cells in contexts of infection, cancer and autoimmunity. Gamma-delta (γδ) T-cells have been recently shown to contain distinct subsets that produce large amounts of either IFN-γ ($CD27^+$) or IL-17 ($CD27\text{-}CCR6^+$). However, the molecular mechanisms that underlie *Ifnγ* and *Il17* gene expression in γδ T cell subsets remain poorly understood. We aim at analysing the miRNA-mediated post-transcriptional regulation of *Ifnγ* and *Il17* expresssion in γδ T-cells.
Materials and methods: γδ T cells were analysed in wild type (wt) and lck-Dicer knockout (ko) mice (Merkenschlager lab). miRNA microarray analysis was performed on sorted peripheral γδ T cell populations ($CD27^+$ and $CD27\text{-}CCR6^+$) from wt B6 mice using the Exiqon miRCURY LNA™ microRNA Array. The expression of candidate miRNAs was validated in thymic and peripheral γδ T cell populations by miRNA RT-qPCR (using LNA primers from Exiqon).
Results: The analysis of lck-Dicer ko mice has revealed a strong impairement in IL-17 (but not IFN-γ) production by miRNA-deficiient γδ T cells. To identify specific miRNAs that might account for this phenotype we performed large scale miRNA studies on the γδ $CD27^+$ and the γδ $CD27\text{-}CCR6^+$ T cell subsets. RT-qPCR validation of two candidate miRNAs, miR-181 and miR-146a, confirmed their up regulation in the γδ $CD27^+$ and γδ CD27-CCR6 subsets, respectively. Interestingly, this differential expression was observed in both thymic and peripheral γδ T cell subsets.
Conclusions: miRNAs are required for the differentiation of IL-17 producing γδ T cells. Specific miRNAs seem to segregate between the IL-17 and the IFN-γ producing γδ subsets both in the thymus and in the periphery. This strongly suggests that the differential expression of miRNAs in γδ T cell subsets is set up in the thymus. Thus, future functional characterization of candidate miRNAs in fetal thymic organ cultures (FTOCs) will be crucial to assess their role in the development of pro-inflammatory γδ T cell-subsets.

P0127

Role of miR-146a-5p in Graft−versus − Host Disease

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Purpose/Objective: A major complication of allogeneic (allo-) haematopoietic stem cell transplantation (HSCT) is Graft-versus-Host Disease (GvHD). GvHD has been classically divided into acute GvHD (aGvHD) and chronic GvHD (cGvHD) depending on the time-point of disease manifestation. Recently, it was shown that miRNAs play a role in GvHD (miR-155) and kidney graft rejection. In this study, we investigated miR-146a-5p which functions in both the innate and adaptive immune system. The aim was to quantify the expression of miR-146a-5p and its validated targets (*TRAF6*, *IRAK1*, *STAT1-α* and *IRF5*) in the whole blood of both aGvHD and cGvHD patients pre- and post-transplantation.

Materials and methods: Whole blood was collected in PAXgene tubes from allo-HSCT patients ($n = 60$) at various time-points pre- (Day-7) and post-transplant (up to 12 months). Expression of miR-146a-5p and its validated targets; *TRAF6*, *IRAK1*, *STAT1-α* and *IRF5* were studied by q-PCR using Taqman miRNA and gene expression assays, respectively. Significance was determined by Mann Whitney *U* test and set at $P < 0.05$.

Results: At 28 days post-transplant, miR-146a-5p expression was significantly down-regulated in II-IV aGvHD patients in comparison to 0-I aGvHD patients ($P = 0.001$). No significant variation was observed between the no cGvHD and cGvHD patient cohort. Likewise, mRNA expression of the targets; *TRAF6*, *IRAK1*, *STAT1-α* and *IRF5* did not vary at any time-point in neither the aGvHD nor the cGvHD cohorts.

Conclusions: We hypothesize that the low expression of miR-146a-5p at 28 days post-transplant in whole blood of II-IV aGvHD patients could be an early indicator of aGvHD severity. We also postulate that miR-146a-5p has a post-transcriptional effect on its targets. This will be elucidated by detection of the targets at the protein level (ongoing).

Poster Session: Myeloid Cell Development

P0129

Cytotoxic CD8$^+$ T cells regulate myelopoiesis via the bone marrow stromal cell niche

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Purpose/Objective: Cytotoxic CD8$^+$ T effector cells (CTLs) are crucial for the protection against primary infection with viruses and other intracellular pathogens. Upon activation by antigen presenting cells, naïve CD8$^+$ T cells undergo clonal expansion, migrate to sites of infection and kill infected target cells via secretion of perforin/granzymes or Fas-ligand/Fas-interaction. However, the complete clearance of pathogens and restitutio ad integrum requires a regulated interplay of innate and acquired immunity. IFN-γ, secreted by activated CTLs, can directly regulate innate immune cells. In addition, IFN-γ was shown to affect hematopoiesis in mice and men; however, these studies yielded conflicting results. Therefore, we analyzed the role of CTL-secreted IFN-γ on hematopoiesis after adoptive transfer of TCR-transgenic T cells and during acute viral infection.
Materials and methods: To assess the effect of activated effector CTLs on myelopoiesis, we infected wild-type mice with the CD8 T cell-controlled lymphocytic choriomeningitis virus (LCMV). In another model, we injected TCR-transgenic p14 CTLs specific for the LCMV-gp33 into mice ubiquitously expressing LCMV-gp33 on MHC class I (H8 mice). Bone marrow (BM) HSCs and myeloid progenitors as well as peripheral blood cells were analyzed at different time points.
Results: Adoptive transfer of activated p14 CTLs into H8 mice did not affect the early hematopoietic stem cell (HSC) compartment but induced the proliferation of multipotent progenitor cells, resulting in increased production of mature myeloid cells. Similar results were obtained with LCMV-infection of wild-type mice. IFN-γ did not affect hematopoietic stem- or progenitor cells directly, but stimulated mesenchymal stromal cells of the BM stem cell niche to produce hematopoietic cytokines including interleukin-6.
Conclusions: BM stromal cells (BMSCs) have an important documented role in the regulation of HSC maintenance and quiescence. Our study demonstrates that BMSCs are central in the regulation of myelopoiesis in response to infection and that CTLs exert an indirect positive feedback on myeloid progenitors during clearance of acute viral infection.

P0130

Detection of TLR agonists by hematopoietic stem and progenitor cells programs macrophage function

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Purpose/Objective: The function of mature macrophages is instructed by signals they receive from factors in their environment and interactions with other cells. For example, Toll-like receptors (TLRs) sense microbial components and the signals they generate coordinate the anti-microbial response. TLRs also detect endogenous danger signals to initiate a response to cell stress and tissue damage. Recent studies showed that TLR expression is not restricted to differentiated cells, but that hematopoietic stem and progenitor cells (HSPCs) also express TLRs. In this study we examined how exposure of HSPCs to TLR agonists alters the function of macrophages derived from them.
Materials and methods: The effects of exposing mouse and human HSPCs to TLR agonists during or prior to macrophage differentiation were examined. The responses of macrophages (adherent, CD11b$^+$ F4/80$^+$ cells) produced by culturing HSPCs with TLR2 and TLR4 agonists ('TLR-derived' macrophages) were compared to those of macrophages derived using M-CSF ('M-CSF-derived' macrophages). The effects of transient exposure of HSPCs to TLR agonists prior to macrophage differentiation driven by M-CSF ('TLR-programmed' macrophages) were also examined.
Results: TLR2 and TLR4 agonists stimulated HSPC proliferation and, depending on the timing, either induced or boosted macrophage differentiation. Upon stimulation of differentiated macrophages, both TLR-derived and TLR-programmed macrophages (mouse and human) exhibited reduced inflammatory cytokine production and oxidative burst activity. However, they were functionally different from 'TLR tolerized' macrophages, which had previously been exposed to TLR agonists but only after macrophage differentiation.
Conclusions: TLR signaling in HSPCs is a previously unrecognized mechanism of regulation of macrophage function, which may be important for limiting the damaging effects of inflammation, but could also impact immune function during/following infection and inflammatory disease. In addition, it might be possible to develop therapeutic approaches based on the concept of TLR programming of HSPCs, which could be applied to the treatment of a variety of diseases, by boosting myeloid cell responses to microbes or tumor cells, or suppressing their inflammatory activity.

P0131

Differentiation of myeloid progenitors is regulated by calcineurin/NFAT signaling

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Purpose/Objective: Haematopoiesis of myeloid cells is a tightly regulated process and complex regulatory mechanisms are in place to control the continuous self-renewal of progenitor cells and their differentiation. The plasticity of the myeloid cell lineage mirrors the diversity of the infectious agents and the diversity of the microenvironments that myeloid cell progenitors are seeding. Indeed the fast replenishment of myeloid progenitors is a key characteristic of innate immunity.
Materials and methods: Different types of haematopoietic progenitors (HSC, MPP, CMP and GMP) were sorted from lineage depleted samples of bone marrow and cultured in presence of SCF, IL-6, Flt3-L, while NFAT signaling was blocked by Cyclosporin A or tacrolimus. Both *in vivo* and *in vitro* proliferation of progenitors were assessed by BrdU staining. Global transcription was analyzed using c-Kit enriched progenitors treated with calcineurin/NFAT inhibitors.
Results: Here we report that calcineurin/NFAT pathway is a key player in the regulation of early stages of myeloid progenitor differentiation from haematopoietic stem cells. We have found a different calcium flux regulation during the development of myeloid progenitors and we have shown that this leads to differential engagement of the calcineurin/NFAT pathway. Our data provide the first evidence that the inhibition of the NFAT pathway *in vivo* enhances the proliferation of granulocyte-monocyte progenitors (GMP). *In vitro*, GMP cells also showed an increased proliferation rate during the differentiation towards dendritic cells when cultured with calcineurin/NFAT inhibitors, such as cyclosporine A and tacrolimus. This regulation can be explained through the interaction of NFAT with cell cycle regulating genes as shown by global transcription analysis of c-Kit enriched progenitors treated with cyclosporin A or tacrolimus.
Conclusions: Taken as a whole our data show that NFAT signaling is a new player in the homeostasis of the myeloid lineage and that immo-

suppressive drugs such as cyclosporin A or tacrolimus can affect the early stages of myeloid haematopoiesis.

P0132
Epigenetic regulation of IRF4 in human monocyte-derived dendritic cells

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Purpose/Objective: Interferon regulatory factor 4 (IRF4) is a well-characterized transcriptional regulator involved in the differentiation of both, T- and B-lymphocytes as well as dendritic cells (DC) and macrophages. Mice deficient in IRF4 are completely devoid of plasma cells and lack functional $CD4^+$ DCs. Although multiple proteins have been shown to regulate IRF4 in different cell-types, including STAT6 (downstream of IL4), c-Rel, JMJD3, the epigenetic regulation of the IRF4 promoter, on the other hand, has received little attention. Here we have studied the activation, epigenetic changes in histone modifications and DNA methylation on IRF promoter during monocyte-derived dendritic cell differentiation.
Materials and methods: Peripheral $CD14^+$ monocytes were purified from the buffy coats of healthy blood donors and differentiated into DCs in the presence of IL4 and GM-CSF. mRNA, chromatin and genomic DNA were extracted from monocytes or DCs to carry out expression analysis, chromatin immunoprecipitation (ChIP) and DNA methylation analysis, respectively. In ChIP and DNA methylation analysis IRF4 promoter was analysed.
Results: We observed a significant increase in IRF4 expression in DCs compared to monocytes. ChIP analysis revealed that DCs had slightly elevated levels of trimethylated lysine in position 4 of the histone H3 (H3K4me3) on IRF4 promoter. More importantly, DCs had significantly lower levels of the repressing histone marker H3K27me3, indicating an active role for lysine demethylase JMJD3 also in human DC-specific IRF4 promoter activation. We also analysed CpG-methylation pattern of IRF4 promoter in monocytes and DCs.
Conclusions: In monocyte-derived dendritic cells IRF4 expression is tightly regulated by the epigenetic modifications in its promoter.

P0133
$Foxp3^+$ Macrophages, do they exist?

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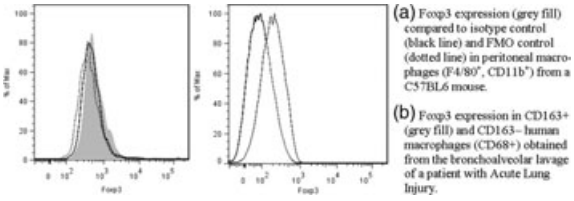
Purpose/Objective: Forkhead box P3 (Foxp3) is the key transcription factor controlling T regulatory cell (Treg) development and function. Foxp3 expression by a subset of immunosuppressive macrophages is highly controversial. We will present data supporting the existence of this suppressive $Foxp3^+$ macrophages in both mice and humans.
Materials and methods: Flow cytometery was used to characterise the $Foxp3^+$ macrophage ($F4/80^+$ $CD11b^+$) cells using a combination of wild type C57BL6, Foxp3 eGFP and Foxp3 GFP DTR mice. Bone marrow derived macrophages (BMDM) were cultured conditions inducing a pro-inflammatory M1 or anti-inflammatory M2 phenotype and the expression of Foxp3 assessed using real time PCR. Mice with a myloid specific KO of suppressor of cytokine signaling proteins (SOCS)2 (M1 phenotype) or SOCS3 (M2 phenoytpe) were used to further assess the phenotype of $Foxp3^+$ macrophages. The expression of Foxp3 in human alveolar macrophages was also examined.
Results: A small but distinct population of $Foxp3^+$ macrophages was consistently identified by flow cytometry. A variety of approaches were used to demonstrate the validity of this population. An increased percentage of $Foxp3^+$ macrophages were detected in SOCS3KO mice which have been previously show to have exclusively M2 macrophages, in contrast no $Foxp3^+$ macrophages were detected in SOCS2 KO (M1) mice. $Foxp3^+$ expression was upregulated in M2 skewing conditions, most strikingly in SOCS3 KO mice. Foxp3 expression was also detected in human M2 ($CD163^+$) macrophages.



(a) Foxp3 expression (grey fill) compared to isotype control (black line) and FMO control (dotted line) in peritoneal macrophages (F4/80$^+$, CD11b$^+$) from a C57BL6 mouse.

(b) Foxp3 expression in CD163+ (grey fill) and CD163− human macrophages (CD68+) obtained from the bronchoalveolar lavage of a patient with Acute Lung Injury.

Conclusions: $Foxp3^+$ Macrophages are a contentious cell population. We have presented evidence that we believe establishes this is a genuine population, as opposed to auto-fluorescence, which is upregulated under M2 conditions. The increased expression of Foxp3 we observed in macrophages from SOC3 KO mice is consistent with previous findings in Tregs, which are deficient in SOCS3 protein expression. Upregulation of SOCS3 in Tregs reduces their suppressive function; we postulate that a similar mechanisms may regulate $Foxp3^+$ macrophages.

P0134
Identification of regulatory elements within the minimal promoter region of PRDM1β in myeloma cells

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Purpose/Objective: PRDM1 is a transcriptional repressor required for the differentiation of plasma cells and short-lived effector T cells. PRDM1 exists as two isoforms, PRDM1α and PRDM1β. The repressor activity is associated mainly to PRDM1α isoform which control key target genes involved in growth control, apoptosis and differentiation. PRDM1β lacks the amino-terminal 101 amino acids of the full isoform PRDM1α which results in a loss of repressive function on multiple target genes. Both proteins are encoded by the same gene but PRDM1β uses an internal alternative promoter. In lymphoma and myeloma cells PRDM1β isoform is up-regulated and it is associated with an increased c-MYC expression. However, little is known about the transcriptional control of the alternative promoter regulating PRDM1β.

For this reason, our principal aim in the present work is to identify 'trans' and/or 'cis' regulatory elements that take part in the transcriptional regulation of this promoter.
Materials and methods: With this purpose different fragments upstream the PRDM1β promoter were cloned in the pGL3-Luc vector. The constructions were transfected in different cell lines and tested for luciferase expression. After that, a new combination of EMSA, SELDI-TOF and Mass Spectrometry techniques were used for the identification of transcription factors which bind to PRDM1β promoter in myeloma cells.
Results: The minimal promoter region with repressor activity was located between -522 to -474 relative to the ATG codon. An analysis of this sequence with Transfactor database revealed a possible binding site for TP53. EMSA assays show at least two prevalent retarded complexes but supershift assays do not identify it as TP53. Mutations affecting the binding site for these complexes increased the promoter activity to basal levels. Binding sites and molecular weights of at least two

transcription factors were defined by EMSA and SELDI-TOF. Actually, we are analyzing the sequence of these transcription factors by Mass Spectrometry.

Conclusions: Our data show a repressor 'cis' element in PRDM1β promoter regulated by at least two transcription factors. Considering that PRDM1β expression indicates poor disease outcome in patients with lymphomas and myelomas, the control of the expression level of PRDM1β in those cells could be used as a therapeutic target of these malignancies.

P0135
Influence of CD137L signaling in myelopoiesis during inflammation

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Purpose/Objective: CD137 is a costimulatory molecule expressed on activated T cells. The signaling of CD137 into T cells upon ligation by its ligand, CD137L, which is expressed on antigen presenting cells (APC), can potently enhance the activation of T cells. It has been shown that CD137 can also induce signaling into APC via CD137L. The signaling of CD137L into the cells it is expressed on is termed reverse signaling to distinguish it from the signaling of CD137. The aim of this project is to investigate the role of CD137L on myelopoiesis under inflammatory condition.

Materials and methods:

1. Acute peritonitis was induced in mice by injection of LPS and *E. coli*. Myeloid population and T cell population were examined in the bone marrow and spleen. Proliferating populations were tracked by BrdU labeling.
2. Activated WT and CD137$^{-/-}$ T cells were cocultured with WT bone marrow cells *in vitro*. Proliferation of cells were determined by CFSE labeling.

Results: Our data show that when stimulated through CD137L murine monocytes become activated as evidenced by increased adherence; release of inflammatory cytokines. *In vivo* CD137 also represents a novel and potent growth and differentiating factor for murine myeloid cells during inflammation. In an acute peritonitis there is a significantly larger increase of myeloid cells, particularly of monocytes, in the bone marrow and spleen of wild type (WT) than of CD137$^{-/-}$ mice. This demonstrates that CD137*CD137L interactions significantly enhance myelopoiesis during inflammation. BrdU labeling experiments revealed that the increase in the number of myeloid cells is due to enhanced local proliferation instead of an influx from other organs. Further investigations revealed that the source of CD137, which drives this enhanced myelopoiesis during inflammation are CD4$^+$ T cells. *In vitro*, WT activated CD4$^+$ T cells induced higher expression of CD11b as well as enhanced proliferation of WT bone marrow cells than CD137$^{-/-}$ activated CD4 T cells, partly through the production of GM-CSF.

Conclusions: These data suggests that CD137$^+$ T cells play a pivotal role in myelopoiesis during inflammation. It is hypothesized that when inflammation is induced, T cells are activated and home to bone marrow and interact with CD137L-expressing progenitor cells and monocytes. Future experiments will focus on examining whether adoptively transferred activated CD4$^+$ T cells can restore the myelopoiesis in CD137$^{-/-}$ mice.

P0136
PAR-2 activation inhibits human osteoclast differentiation

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Purpose/Objective: Osteoarthritis (OA) is a joint disease characterised by both cartilage degradation and increased subchondral bone formation (osteosclerosis). Osteoblasts and osteoclasts are the cells responsible for the maintenance of bone homeostasis[1]. As osteoclasts resorb bone, reduction in their number or function may be responsible for osteosclerosis in OA. PAR-2 is a G-protein coupled receptor known to be involved in inflammation. PAR-2 has recently been shown to play a critical role in experimental OA as a reduced disease phenotype occurs in mice lacking this receptor [2]. This study seeks to identify a role for PAR-2 in human osteoclast differentiation.

Materials and methods: CD14$^+$ monocytes were purified from the peripheral blood of healthy subjects and cultured for 14 days with M-CSF and sRANKL to promote osteoclast differentiation. In parallel experiments, osteoclast differentiation was performed in the presence of a selective PAR-2 activating peptide, SLIGKV-NH_2, or the reverse sequence peptide (RP) and the number of osteoclasts quantified by counting the TRAP$^+$ multinucleated ($\geq$3) cells in each well.

Results: CD14$^+$ monocytes have low levels of surface PAR-2, with cultured pre-osteoclasts displaying denser PAR-2 expression. Differentiation of pre-osteoclasts in the presence of SLIGKV-NH_2 resulted in a significant decrease ($P < 0.01$; $n = 3$) in osteoclast formation (80.8 ± 28.7; mean ± SEM) compared to vehicle (473 ± 68.8) or RP-treatment (481.8 ± 90.7; $P < 0.05$).Importantly, the PAR-2 activating peptide did not affect cell viability.

Conclusions: These findings suggest that PAR-2 activation can inhibit human osteoclast differentiation, potentially promoting osteosclerosis as observed in OA. This corroborates a previous study in murine cells [3] and importantly translates this finding to human osteoclast differentiation.

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P0137
Role and function of microglia cells in autoimmune CNS inflammation

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Purpose/Objective: Unlike other glia cells, microglia originate from the myeloid cell lineage and therefore often are considered as resident brain macrophages. They act in the first response to direct injury or peripheral insults. However, the exact role microglia play during the process of autoimmune disease is not clear yet. Using the iDTR system microglia were depleted *in vivo*. After optimizing the efficiency of the depletion system, the role of microglia in health and disease (EAE) was analysed.

Materials and methods: The new microglia/ macrophage specific CX_3CR1^{CreER} strain was crossed to iDTR mice. Administration of tamoxifen lead to Cre-mediated excision of a loxP flanked DNA fragment in both cell types. Whereas macrophages have a fast turnover and are replaced by unaffected precursors, the microglia persist in the

modified stage. By crossing the CX_3CR1^{CreER} mice to the Rosa26-RFP reporter line we could investigate the efficiency of the system.

Then the CX_3CR1^{CreER} mice were crossed to iDTR mice to specifically express the diphtheria toxin receptor in microglia. To optimize the system for efficient microglia depletion we tested different DT administration protocols.

Results: By analyzing the reporter expression after tamoxifen treatment in the age of 2 weeks, mature mice exhibit no RFP positive macrophages whereas 70% of the microglia still expressed RFP. This phenotype was also observed during EAE progression.

With two Tamoxifen and three successive DT injections we could achieve an efficiency of 80% microglia cell ablation. Interestingly, we observed by histology 50% of repopulating microglia already 6 days after depletion. Approximately 2 weeks after depletion the microglia numbers were even increased compared to unaffected controls. In FACS analysis we found a CD45.2 high $Ly6C^+$ population on day 6, indicating a repopulation of microglia by peripheral monocytes.

During EAE the mice showed a more severe course of disease and exhibit a neurological phenotype.

Conclusions: Mice without microglia sustained a more severe EAE course, which was accompanied by showing neurological deficits. Furthermore microglia were rapidly repopulated after depletion and therefor they seem to play a critical role in homeostasis of brain functions and environment.

P0138

T cell immunoglobulin and mucin family members are expressed by hematopoietic cells, especially developing macrophages, in mouse and chicken embryo

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Purpose/Objective: T cell immunoglobulin and mucin domain containing (TIM) molecules are known regulators of the immune response, e.g. in asthma and autoimmunity. In adult mouse, TIM4 is known to be expressed by antigen presenting cells and TIM1, TIM2 and TIM3 by T cells in which they regulate the balance between T_{H1} and T_{H2} responses. In an earlier study, we reported *tim1* and *tim4* to be expressed differentially by $CD45^+$ hematopoietic cells in para-aortic (PA) region of chicken embryo. Chicken PA-region, as mouse fetal liver (FL), provides a microenvironment for HSC to further proliferate and differentiate into hematopoietic progenitor cells (HPC). Our aim is to study the expression and role of TIM molecules in fetal hematopoiesis.

Materials and methods: We have analysed TIM expression in chicken by PCR and *in situ* hybridization and in mouse by qPCR and FACS staining. We have also characterized mouse FL TIM expressing cells with antibodies to identify their developmental stages.

Results: PCR analysis of chicken embryonic day 4 (ED 4), ED 7 and ED 13 tissues revealed *tim1* to be expressed more widely in younger embryos, especially in PA-mesenchyme which is a known site for HSC differentiation and development. Chicken *tim4* transcripts were amplified from almost all studied embryonic tissues. It«s wide expression pattern was also evident in *in situ* hybridization suggesting *tim4* to be expressed by macrophages during development. In 12.5 dpc mouse, *tim3* and *tim4* transcripts were expressed highest in FL and mostly absent in non-hematopoietic tissues. *tim1* expression was shown to be high in lung.

Further characterization of 13.5 dpc mouse FL cells showed TIM4 to be expressed by $CD45^+$ $F4/80^{high}$ cells which are presumably macrophages. $TIM4^+$ $CD45^+$ $F4/80^{high}$ cells included populations expressing stem cell markers c-kit and/or SCA-1. TIM1 and TIM3 were also shown to be mainly expressed in $CD45^+$ hematopoietic cells. Both $CD45^+$ $TIM1^+$ and $CD45^+$ $TIM3^+$ populations included a subpopulation of $F4/80^+$ $c\text{-}kit^+$ immature macrophages. $CD45^+$ $TIM1^+$ $F4/80^-$ and $CD45^+$ $TIM3^+$ $F4/80^-$ cells contain few $c\text{-}kit^+$ and/or $SCA\text{-}1^+$ cell, possibly HSC or HPC.

Conclusions: In summary, TIM family molecules are expressed by mouse and chicken embryonic hematopoietic tissues. In mouse FL, TIM1, TIM3 and TIM4 expression was shown in hematopoietic cells, especially in immature macrophages suggesting a possible role in hematopoiesis.

P0139

T-cell specific deregulation of Notch3 receptor induces alterations in the development of myeloid compartment unveiled by the deletion of NF-kappaB/p50 expression

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Purpose/Objective: T-cell specific deregulation of Notch3 in transgenic mice (*N3-tg*), induces the development of a T-cell acute lymphoblastic leukemia (T-ALL), sustained by the constitutive activation of NF-kB canonical pathway. Besides, Notch signalling modulation in bone-marrow stromal cells or in hematopoietic stem cells, has been related to alterations in differentiation/proliferation processes of myeloid cells. To clarify the Notch/NF-kB relationships in the progression of T-ALL and the effects of a T-cell specific deregulation of Notch on myeloid compartment, we decided to delete NF-kB canonical pathway in *N3-tg* mice.

Materials and methods: We generated *N3-tg/p50*$^{-/-}$ mice, deleted of the NF-kB/p50 subunit in a Notch3 transgenic background. The follow-up of double mutant versus *N3-tg* mice versus relative controls was conducted and immunophenotyping of hematopoietic cell subsets was performed at different age and in multiple tissues from the indicated animals by flow-cytometry tecniques. Total RNA and protein extract samples, derived from sorted T- or myeloid-cells of our mice models, were processed for RT-qPCR and Western blotting analysis, respectively, to test the expression of Notch-related molecules.

Results: The progression of T-ALL, as defined by the peripheral expansion of immature $CD4^+$ $CD8^+$ T cells, was strongly inhibited in *N3-tg/p50*$^{-/-}$ versus *N3-tg* mice. However, the double mutant mice succumb earlier than *N3-tg* counterparts displaying a dramatic increase of $Mac1^+$ $Gr1^+$ myeloid cells in both spleen and blood, as well as of granulocyte/monocyte progenitors in the bone marrow. The expansion of myeloid subsets was detectable at a lower extent also in *N3-tg* versus *wild-type* mice. Preliminary data indicate that $Mac1^+$ $Gr1^+$ cells do not express Notch3, suggesting that this receptor may influence the equilibrium of the myeloid compartment mainly *in trans*, possibly through its interaction with the Jagged-1 ligand.

Conclusions: Our results suggest that the NF-kB canonical pathway deletion inhibits the T-ALL progression, thus unveiling the influence of Notch signalling modulation on the behaviour of myeloid cells. We provide a useful model to extend our understanding of Notch/NF-kB interplay in driving the relationships between lymphoid and myeloid compartments in the context of hematological malignancy.

P0140

The Frizzled-like domain from Collagen XVIII (FZC18) controls hematopoiesis and myeloidogenesis *in vivo*

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Purpose/Objective: Collagen XVIII (Col18), characterized by the antiangiogenic / tumor-suppressive endostatin domain at its C-terminus, occurs as three N-terminal variants. The corresponding gene has two promoters, promoter 1 encoding the shortest variant and promoter 2 the middle and longest variants. The longest variant is characterized by a frizzled-like domain (FZC18), known to be shed from full-length Col18, and shown to act as a soluble inhibitor of the Wnt3a/b-catenin pathway. In view of the known importance of Wnt3a in hematopoietic stem cell (HSCs) development and myeloidogenesis, and the fact that Col18 is expressed in the bone marrow, we decided to investigate the effects of lack of Col18 on mouse hematopoiesis.

Materials and methods: Using total- and promoter-specific knockout mice for Col18, the HSCs isolated from adult bone marrow were fractionated into KLS and srHSCs populations basing on their expression of CD34, a master marker for HSCs/ multipotent progenitors discrimination. The same mouse cohorts were also screened for differences in peripheral leukocyte counts. Moreover, 12.5 and 13.5 days p.c.-old embryos were collected and HSCs in the liver screened for CD41 (a marker for yolk sac-derived HSCs) and CD45 (a marker for liver HSCs) expression.

Results: Our data show for the first time a role for Col18 on mouse hematopoiesis, as witnessed by a significant increase in HSCs in the knockout mice, and consequently a wide increase in monocyte and granulocyte counts. This effect was found to be specific for promoter 2 knockout mice.

Conclusions: Specific ablation of the middle and longest Col18 variants leads to an increase in srHSCs which reflects on peripheral monocyte and granulocyte accumulation, without any appreciable effect on the specific compartment of the immune response. These data thus provide evidence of novel roles for Col18 and possibly its FZC18 variant, capable of binding Wnt molecules, as endogenous controllers of myeloidogenic commitment during HSCs development.

P0141

The role of microRNA in microglia maintenance and function

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Purpose/Objective: Microglia are macrophages populating the central nervous system (CNS). They continually survey the CNS and mediate phagocytosis during steady state (development, synaptic pruning) and contribute to neurodegenerative diseases. To protect the CNS from harmful inflammation, microglia activity is tightly regulated by various factors including microRNAs (miRs). To study microglia in context we take advantage of CX_3CR1-CreER mice that harbor a latent recombinase inducible upon drug application. In tamoxifen-treated mice, rearrangements in peripheral CX3CR1-expressing mononuclear phagocytes are progressively lost due to their renewal from BM-derived precursors. In contrast, the self-maintaining CX_3CR1^+ microglia remain permanently genetically manipulated. Here, we generated *in vivo* conditional miR deficiency in microglia by crossing CX_3CR1-CreER mice with mice harboring a conditional allele of Dicer, the miR processing enzyme yielding MG^{DDicer} mice.

Materials and methods: Flow cytometry measuring microglial numbers and activation; immuno-histochemistry assessing neurodegeneration, neuroinflammation and astrogliosis; behavioral studies measuring motoric functions.

Results: MG^{DDicer} mice display a progressive severe loss of microglia which is more pronounced in spinal cord than the brain. The remaining dicer-deficient microglia had elevated surface expression of CD11b and CD45, as well as CD86. Immunohistochemistry of microglia showed a classical pattern of activated microglia with increased cell body and amoeboid structure. Finally, preliminary behavioral locomotor studies revealed reduced motoric activity of MG^{DDicer} mice, when compared with their littermates.

Conclusions: Here we establish a critical role of miRNA in the maintenance of microglia. Spinal cord microglia are more sensitive than brain microglia, resulting in lower limb motoric deficiency. Ongoing experiments address whether motor neuron defect results from microglia activation and neuro-inflammation. Moreover, we are in the process of generating mice with deficiencies of specific candidate miRs to define the miR control of microglia homeostasis.

Poster Session: NK Cells

P0142
A new mechanism of resistance to NK cell attack in melanoma

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Purpose/Objective: The efficiency of human NK cells in eliminating tumors is generally assessed by measuring their killing capability against a large number of tumor cell lines in cytolytic assays. The E/T ratios used *in vitro*, however, may be not consistent with the levels of NK cell infiltration of tumors. In this study, by the histological analysis of 28 melanoma samples, we first assessed the level of NK infiltration at the tumor site, and then set up NK-melanoma cell co-culture experiments in order to assess the possible functional interactions occurring between the two cell types at such low E/T ratio.
Materials and methods: Melanoma cells recovered from co-cultures were analyzed by FACS and in cytolytic assays in order to assess possible changes in their surface phenotype and their susceptibility to NK cell mediated lysis.
Results: The histological analysis of melanoma samples revealed that the NK/Melanoma cell (NK/M) ratio *in situ* is far lower than that generally used *in vitro* to evaluate the NK cell killing capability. In co-culture experiments performed at similar low NK/M ratio, we observed that a large part of melanoma cells survived and, more importantly, acquired strong resistance to NK cell-mediated killing. This effect was essentially due to an increased surface expression, in melanoma cells, of classical and non classical HLA-I molecules and was dependent on NK-mediated IFN-g release. NK-conditioned Melanoma cells also showed slight expression decreases of different NKG2D-Ligands and of GPR56 (a possible receptor for ECM components) but these changes didn't apparently affect melanoma cell susceptibility to NK cells. Further analysis on melanoma lesions revealed a higher HLA-I expression on tumor cells that were proximal to NK cell infiltrate thus suggesting that the acquisition of resistance could actually occur *in vivo*. Analysis of different NK-activating cytokines indicated that IL-15 might in part overcome this new phenomenon of tumor resistance.
Conclusions: This study provides new elements for a proper evaluation of NK cell efficacy to fight cancer *in vivo* and proposes IL-15 as valuable element to prevent from this newly described phenomenon of tumor resistance.

P0143
Accelerated effector: target conjugate formation and granule polarization in leukotriene B4-treated human NK cells

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Purpose/Objective: The natural killer (NK) cell is an important element of innate and adaptive immunity due to its strong cytotoxic response and cytokine production. Previously, we determined that leukotriene B_4 (LTB_4) augmented NK cytotoxicity and characterized the expression and contribution of LTB_4 receptors on NK cells. However, the mechanism of LTB_4-induced cytotoxicity augmentation is not yet clear. The killing of target cells by NK cells involves recognition, conjugation between NK and target cells, granule polarization and degranulation. Thus, we sought to investigate the effect of LTB_4 at these stages of cytotoxicity.
Materials and methods: Peripheral blood mononuclear leukocytes and purified NK cells were isolated from healthy donors. Calcium influx was measured with Fluo-4 stain. LFA-1 activation was determined with a specific antibody. The NK-target conjugates were determined as $CD56^+$ $CFSE^+$ after co-incubation of NK cells ($CD56^+$) and K562 cells ($CFSE^+$). CD107a (LAMP1) was chosen as marker for degranulation. The kinetics of granule polarization was evaluated by confocal microscopy in NK92MI. Cytotoxicity was measured with the 7AAD stain by flow cytometry.
Results: LTB_4 induces concentration-dependent calcium influx in NK cells. NK-target binding increases 1.5-fold after a 5-min stimulation with LTB_4, however, this increase is transient and returns to control levels within 30 min. In NK92MI cells, LTB_4 results in granule polarization to the synapse area within 12 min after NK-K562 conjugation, compared to 20 min in unstimulated cells. In addition, higher CD107a expression on the NK cell surface is observed within 30 min of stimulation with LTB_4.
Conclusions: Our findings suggest that a more rapid NK-target conjugation, granule polarization and increased degranulation may contribute to LTB_4-induced augmentation of NK cell cytotoxicity.

P0144
Acute myeloid leukemia cells evade from NK cells anticancer response by forming an impaired immunological synapse

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Purpose/Objective: Natural Killer cells (NK) cells are largely studied as effectors cells in the anticancer immune response in patients with Acute Myeloid Leukemia (AML). AML blasts were shown to express ligands for NK cell activating receptors. We and others observed some defects in the expression of major activating receptors including NKG2D, DNAM-1 and NKp46, on patients NK cells. However little is known about the direct interaction between NK cells and leukemic cells.
Materials and methods: We used primary NK cells from healthy donors and AML cell lines THP1 and HL60, in comparison to the highly sensitive NK target K562. Interactions between NK and tumor cells were evaluated by flow cytometry and confocal microscopy, focusing on the polarization of actin, CD107a and LFA-1 toward the immune synapse. Long-term impact of the NK-AML tumor contact was evaluated after *in vitro* co-cultures.
Results: NK cells formed less conjugates with HL60 than with THP1, and in both cases less than with K562. Upon contact with THP1 and HL60, first steps of the lytic synapse were established: actin polymerized and the adhesion molecule LFA-1 was recruited to the contact area. However lytic granules polarization toward the synapse was defective: average percentage of conjugates showing polarized CD107a was 32% and 29% with THP1 and HL60 respectively, compared to 55% with K562 ($P = 0.001$ and 0.0007 respectively). Interactions with HLA-Cw*03:04 transfected and untransfected K562 lines were not different, ruling out a primary effect of inhibitory receptor ligands.

After 48h co-culture of PBMC with AML cell lines, the expression of DNAM-1 on all NK cells was down-regulated with both THP1 and

HL60. NKG2D expression was reduced only with THP1 and the decrease of NKp46 was significant on the CD56Bright subset only with both cell lines. NK cells retrieved from co-culture with THP1 showed a decreased activity in a CD107a degranulation assay (16.8% degranulation of THP1 co-cultured NK cells versus 43.4% for control cultured NK cells, $P = 0.03$).

Conclusions: Our observations show defects in lytic granule polarization and function during the early interaction between NK cells from healthy donors and AML cell lines. This engagement leads also to a decreased expression of activating receptors and thereby to sustained cytolytic activity impairment.

P0145

Alloreactive NK cells in haploidentical-HSCT (HAPLO-HSCT) upon infusion of either CD34$^+$ OR TCR alpha beta/CD19-depleted cells

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Purpose/Objective: We aimed to analyze the NK cell repertoire in pediatric leukemia patients transplanted from alloreactive haploidentical donors, comparing two different procedures: infusion of either CD34$^+$ or TCR $\alpha\beta$/CD19-depleted cells. This second graft manipulation provides a pool of stem cells in addition to effectors cells, potentially active against infections (i.e. NK and TCR $\gamma\delta$ T lymphocytes) and against leukemia cells (i.e. alloreactive NK cells).

Materials and methods: Clinimacs technology was used for either the positive selection of CD34$^+$ or the negative selection of $\alpha\beta$ T cells and CD19 B cells. The analysis of donor KIR gene profile and HLA-I typing of both donor and recipient may predict, in the case of KIR/KIR-ligand mismatch in GvH direction, the existence of alloreactive NK cells. Combinations of anti-KIR mAbs were used to define the size of alloreactive NK cell subset and the expression of activating KIR.

Results: Genetic, phenotypic and functional analyses have been performed to study the NK cell repertoire. In some cases, when two NK alloreactive donors were available, we have selected the most appropriate one taking into account features which have been correlated with a better clinical outcome (KIR genotype B/X better than A/A, higher B content score, larger size of alloreactive subset), considering the positive role that activating KIR can exert. Indeed, we have demonstrated that the presence of KIR2DS1 in C1$^+$ /C2$^+$ donors can be relevant in the lysis of C2$^+$ /C2$^+$ leukemias. At the 1st month post-transplant, while NK cells in CD34$^+$ grafts predominantly displayed an immature phenotype, in TCR $\alpha\beta$/CD19-depleted grafts we observed a high proportion of NK cells with a mature phenotype (CD56^{dim}, KIR$^+$/NKG2A$^-$ and CD57$^+$) and a detectable alloreactive NK cell subset.

Conclusions: These preliminary data suggest that, in patients transplanted with TCR $\alpha\beta$/CD19-depleted cells, mature donor-derived NK cells, including alloreactive NK cells, persist at 1st month. Longer follow-up and larger group of patients are necessary to clinically assess the potential beneficial effects of this graft manipulation in the control of infections and leukemia relapse.

P0146

Altered number and function in different subpopulations of NK cells from elderly

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Purpose/Objective: The process of immunosenescence affects both innate and acquired immune response. Previous studies show that NK cells are affected by ageing. The aim of this work is to study *in vitro* the effect of age on the phenotype and function of NK cell subpopulations through the quantification of the basal content of granzymes A and B.

Materials and methods: To determine *in vitro* the frequency of NK cells and their subpopulations, 32 young donors and 20 elderly donors were included. In order to analyze the changes associated with aging in the intracellular expression of granzymes on NK cells subpopulations, defined by the expression of CD56/CD16, the basal content of granzyme A ($n = 33$ young and 23 elderly donors) and granzyme B ($n = 29$ young and 24 elderly donors) was determined by multiparametric flow cytometry.

Results: The frequency of subpopulations CD56brightCD16- and CD56brightCD16low of the elderly compared with young people was decreased while the subpopulation CD56-CD16$^+$ was increased. The percentage of NK cell subpopulation CD56dimCD16$^+$ was maintained. Our results showed an increased expression of granzyme A and B in total NK cells from elderly individuals when compared with young donors. In relation with the NK cells subpopulations, we found an increased expression of granzyme A and granzyme B in CD56bright-CD16- and CD56brightCD16low of elderly relative of young people, while it was decreased in CD56dimCD16$^+$ of the elderly compared with young people. Similar expression rates of granzyme A and B were found in the subpopulation CD56-CD16$^+$ from elderly and young donors.

Conclusions: The results indicate that NK cells are among the lymphocyte populations affected by the process of 'immunosenescence' in phenotype and function. These changes affect differently the different subpopulations of NK cells. The CD56bright subpopulation is most affected in this process, becoming more cytotoxic capacity with age.

P0147

CD161 expression defines a phenotypically, functionally and transcriptionally distinct set of NK cells which are specifically depleted in HIV infection

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Purpose/Objective: HIV-1 infection leads to a gradual loss of natural killer cells, which correlates with disease progression. CD161 is a C-type lectin molecule expressed on the majority of NK cells. CD161$^+$ NK cells have been previously observed to be reduced in HIV infected patients; however functional analyses according to CD161 expression have not been performed. In this study we have characterized the unique properties of NK cells expressing CD161 by defining their phenotype, transcriptional signature, and functional capacity in healthy donors, and for the first time present longitudinal data showing CD161 expression recovering with HAART treatment in chronic HIV patients.

Materials and methods: $CD161^+$ and CD161- NK cells were sorted from PBMCs isolated from healthy adult blood, and expression profiling was performed using the Agilent platform. Differentially expressed genes and functional properties were analysed using FACS and intracellular cytokine staining. The flux of these cells in chronic HIV was analysed using longitudinal samples from the Swiss HIV cohort study.
Results: We report that CD161 is expressed on the majority of $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells in adults (average 80.57% and 89.79%, respectively). $CD161^+$ NK cells are enriched for activating NK cell receptors and cytolytic molecules while CD161- NK cells express inhibitory NK cell receptors. Furthermore, there was a significant enrichment of cells producing IFNγ and with greater proliferative capacity in response to target cells and cytokines in the $CD161^+$ NK cell subset compared to the CD161-NK cell subset. Intriguingly, the frequency of $CD161^+$ NK cells was dramatically lower in HIV patients before HAART treatment compared to healthy controls (62.83% and 89.37% respectively, $P < 0.0001$), but showed a stepwise increase their CD161 expression after HAART initiation, correlating with a higher CD4 count ($r = 0.5013$, $P = 0.0107$).
Conclusions: These results suggest that CD161 expression on NK cells is a marker of a more activated and functional subset within both the $CD56^{bright}$ and $CD56^{dim}$ NK populations, displaying enhanced antiviral function. This highly functional subset of NK cells is reduced in chronic HIV, but recovers with HAART. Further understanding of the role of this NK cell subset in disease progression may provide new insights into HIV pathogenesis.

P0148
Characterization of mice with a deficiency in natural killer cell missing self reactivity

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Purpose/Objective: In experiments with a KO mouse strain deficient for a nonclassical MHC class I like gene, but with otherwise normal MHC class I expression, we unexpectedly observed that the mice had severely impaired capacity for NK cell mediated rejection of MHC class I deficient cells. Control experiments with independently derived KO strains for the same gene as well as F1 and F2 intercrosses with B6 mice, indicated that the NK cell defect was not caused by the targeted gene and the two phenotypes segregated independently. This mouse strain represents an opportunity to study the mechanisms and regulation of missing-self rejection and other NK functions.
Materials and methods: *in vivo* studies of NK cell mediated rejection of normal and tumor cells. *In vitro* flow cytometry based assays to study the phenotype and functional capacity of NK cells. *In vitro* cytotoxicity assays. Construction of bone marrow chimeras.
Results: The mouse strain showed a normal number of NK cells and normal profiles for their cell surface phenotype. *In vivo*, the NK cells showed a severely impaired capability for missing self rejection of normal spleen (b_2m- versus b_2m^+) and tumor cells (RMA-S versus RMA). Kinetic studies and tumour outgrowth experiments revealed a profound defect rather than a delay in missing-self rejection. In contrast, there was a normal or partly impaired capacity with respect to other NK functions, such as positive allorecognition, CD107a/IFNγ production and ADCC. Genetic crosses suggested that the defect is under control of (a) recessive gene(s). Bone marrow chimeras indicated that the defect is bone marrow derived and not due to the host environment..
Conclusions: Our results indicate that this mouse strain has a genetically based, selective deficiency in the killing pathway used to sense missing self. This defect is intrinsic to bone marrow cells, possibly to the NK cells themselves. One possibility is a dysfunction that involves an unknown activating receptor or signaling molecule, most critical for missing self recognition. Alternatively, the defect may disturb education/licensing for missing self recognition, while leaving other NK functions relatively intact. Further studies of these mice may shed light on the regulation and the *in vivo* role of missing self recognition as compared to other NK cell functions, in different responses or diseases.

P0149
Characterization of NK cells infiltrating metastatic melanoma lymph nodes

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Purpose/Objective: Over the past decades, the incidence of melanoma has been constantly increasing. Malignant melanoma has a high metastatic potential and is difficult to cure. There are numerous arguments indicating that melanomas are immunogeneic and that the immune system can control the tumor growth in certain conditions. We focus our interest on Natural Killer (NK) cells, potent cytotoxic effectors that can control metastases in several murine tumor models.
Materials and methods: To study the NK cells that infiltrated lymph nodes, an early site of metastasis in melanoma, we have set up experimental methodology to characterize NK cells in metastatic lymph nodes (LN) from 25 stage III melanoma patients. We have analysed *ex vivo* by multicolor FACS analyses and functional assays in response to K562 stimulation the NK cells infiltrating lymph nodes. The tumor burden in the samples was estimated by the pathologist to determine the level of tumor invasion of the LN and distinguish metastatic (M) and non metastatic (nM) LN. We included mediastinal LN from five donors as controls.
Results: We first show that the $CD56^{dim}$ NK subset major circulating NK subset is less represented in LN. Our preliminary data show that compared to normal LN, the phenotype of NK cells from patient derived LN is different: however, NK cells from MLN and nMLN exhibit close phenotype and are characterized by increased expression of certain activating receptors. Chemokine receptors are also expressed by LN infiltrating NK cells from patients suggesting their migration from the periphery. LN infiltrating NK cells from patients are also characterized by decreased functionality in response to strong stimulation (PMA/$Iono^+$K562). We also determine the frequencies and distribution of NKp46 positive NK cells by immunohistochemistry in a series of sentinel lymph nodes from melanoma patients.
Conclusions: These results constitute a complete *ex vivo* characterization of NK cells infiltrating LN from melanoma patients.

P0150
CXCL8 production and absence of LFA-1 surface expression identify human NK cells at early stages of differentiation characterized by CD161 molecule with activating function

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Purpose/Objective: Human NK cell development is a step-by-step process characterized by phenotypically identified stages. To define

factors that involved *in vitro* NK cell differentiation and maturation from $CD34^+$ progenitors, we performed time course analysis of cytokine release and of acquisition NK cell markers expression.

Materials and methods: Umbilical cord blood $CD34^+$ stem cells were cultured *in vitro* with IL-7, IL-15, IL-21, SCF and Flt3-L in. Leucocytes were analysed for informative cell surface markers by cytofluorimetric assays and separated by cell sorting according to CD33, CD161, CD56 and LFA-1 surface expression. Cells were tested for cytolytic activity and/or cytokine production. Cytofluorimetric assays were used to analyse CD161-mediated signal transduction, cytokine or CD107 expression. We performed Real Time PCR for detection of CXCL8 mRNA.

Results: We demonstrated that LFA-1 expression allows discriminating between immature non-cytolytic $CD161^+$ $CD56^+$ LFA-1- NK cells (immNK) and more differentiated cytolytic $CD161^+$ $CD56^+$ $LFA\text{-}1^+$ NK cells (difNK). LFA-1 expression allowed distinguishing between $CD161^+$ $CD56^+$ LFA-1- immNK cells producing large amounts of CXCL8, but not IFN-γ, and $CD161^+$ $CD56^+$ $LFA\text{-}1^+$ difNK cells, producing IFN-γ, but not CXCL8. We could also identified *in vivo* immNK cells able to produce CXCL8. Indeed, CXCL8 mRNA expression was detected in fresh tonsil Lin-$CD56^+$ $CD117^+$ CD94/NKG2A- stage III immature NK cells and these cells expressed CXCL8 protein upon PMA stimulation. CXCL8 release was also induced upon crosslinking of NKp44 and CD161. The activating function of CD161 was confined to immNK cell subset, since it did not induce cytokine release or CD107a expression in $CD161^+$ $CD56^+$ $LFA\text{-}1^+$ difNK cells or in mature PB NK cells. CD161 crosslinking in immNK cells induced increase of Akt-pS473 expression and CD161-mediated CXCL8 expression was significantly reduced by the presence of Akt inhibitor XI. The addition of anti-CXCL8 neutralizing antibody led to a partial inhibition of NK cell differentiation suggesting a regulatory role of CXCL8 during early NK cell differentiation.

Conclusions: Altogether, these data provide novel information on NK cell development and may also offer clues to optimize NK cell maturation in hematopoietic stem cell transplantation.

P0151

De novo expression of CCR7 and acquisition of migratory properties by human $KIR2DS1^+$ NK cells interacting with allogeneic $HLA\text{-}C2^+$ monocyte-derived DC or T cell blasts

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Purpose/Objective: In the present study, we investigated the ability of $KIR2DS1^+$ NK cells to de novo express CCR7 after interaction with allogeneic $HLA\text{-}C2^+$ mature dendritic cells (mDC) and T cell blasts.

Materials and methods: In order to better understand the correlation between KIR2DS1-mediated signaling and CCR7-expression, we used mDC from allogeneic donors expressing HLA-C alleles belonging to the C2-specificity and selected alloreactive NK cell clones expressing the activating C2-specific KIR2DS1 receptor and NKG2A (or, as control, clones expressing only NKG2A).

In co-culture experiments, NK cells were mixed with these targets at an effector-to-target cell (E/T) ratio of 1:1 in the presence or in the absence of anti-HLA-Cw4 or anti-NKG2A mAbs and incubated for 1 h at 37¡ C in 5% CO_2. Next, NK cells were directly assessed for surface phenotype, cytolytic activity, migration capacity and cytokine production.

Results: In transplantation, alloreactive NK cells by killing recipient DC play a crucial role in preventing donor's T cell priming and subsequent GvHD. We showed that alloreactive KIR^+ NK cells may *de novo* express CCR7 upon co-culture with allogeneic mDC. The acquisition of CCR7 by NK cells is due to receptor uptake from $CCR7^+$ cells and is negatively regulated by inhibitory KIR-mediated recognition of HLA class I molecules.

Recent data indicate that, KIR2DS1 expression confers alloreactive NK cell responses with potent cytotoxicity against $HLA\text{-}C2^+$ mDC and T cell blasts.

In this study, we analyzed the effects mediated by alloreactive $KIR2DS1^+$ NK cells in the acquisition of CCR7 upon interaction with $HLA\text{-}C2^+$ $CCR7^+$ cells. Analysis of NK cell clones revealed that NK cells expressing this activating KIR acquire CCR7 and that this mechanism is positively regulated by recognition of KIR2DS1-ligand on both allogeneic DC and T cell blasts.

Conclusions: These data may have important implications in haploidentical hematopoietic stem cell transplantation, in which donor $KIR2DS1^+$ NK cells acquire the ability to migrate to lymph nodes, where they can kill recipient's DC and T cells thus preventing GvH (and HvG) reactions.

P0152

Differential TLR3 expression and function in human newborns and adults

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Purpose/Objective: TLR3 is an innate immune system receptor that recognizes dsDNA and is responsible for viral sensing. Human newborns are more susceptible to pathogens then adults, including to viral infections, e.g. herpes simplex type 2. We therefore hypothesized that TLR3 expression, as well as of some other viral-sensing receptors, might differ between newborns and adults.

Materials and methods: We analyzed different leukocyte subsets of cord blood and adult blood, and measured TLR3 mRNA expression in the cells that showed the highest TLR3 expression, i.e. natural killer (NK) and T cells, as well as in placental NK cells. Also, functional characteristics of cord and adult blood NK cells in response to TLR3 ligands were analyzed and compared.

Results: We found a marked difference in TLR3 mRNA and protein expression in cord blood NK and placental NK cells compared to adult blood NK cells. The mRNA expression of TLR7, TLR8, TLR9, MDA-5, RIG-I, PKR and IFI-16 genes for viral-sensing receptors did not significantly differ between newborns and adults. Furthermore, cord and adult blood NK cells differed in their cytotoxicity and the ability to produce IFN-γ as a result of TLR3 activation, showing a functional discrepancy between newborn and adult NK cells.

Conclusions: Our data imply that cord and adult blood NK cells have different potential in the aspect of TLR3 activation which is manifested in a functional divergence of TLR3-mediated responses to viruses between newborns and adults. This contributes to a better understanding of immature immune system of newborns and consequently their higher vulnerability to viral infection.

P0153

Elucidating the role of the NK killer receptor Ncr1 in controlling melanoma using in-vivo reflectance confocal microscopy

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Purpose/Objective: Natural killer (NK) cells play a crucial role in the early host response against viruses, bacteria and malignancies by lysing infected or transformed cells. NK cells are controlled by signals generated upon the engagement of various ligands to inhibitory and activating receptors. Among the later are proteins such as NKG2D, 2B4 and the natural cytotoxicity receptors [NCR (NKp44, NKp30 and NKp46)]. NKp46 is expressed almost exclusively on NK cells, and it is the only NK cell receptor with a mouse orthologue, the Ncr1.

Recently we demonstrated that Ncr1 plays an important role in regulating the growth of melanoma cells and their capacity to metastasize. However, the mechanism by which this is done is yet unclear.

In-vivo reflectance confocal scanning microscopy (RCM) is a novel, non-invasive imaging technique that permits real time visualization of cellular structures in the skin at a resolution close to that of conventional histology. It has been widely used in the diagnosis of both benign and malignant tumors in humans. Only few reports are available on the use of RCM in mice melanoma models.

Objectives(i) To asses the usefulness of *in vivo* RCM not only as a diagnostic tool in humans, but as a research tool capable of imaging mouse model malignancies. (ii) To clarify the mechanism by which Ncr1 is involved in melanocytic tumors and metastases development in mice.

Materials and methods: Using B16F10.9 tumors and an Ncr1 knockout mouse generated by our group we checked various melanoma indices of tumor development by means of RCM.

Results: (i) We determined that there is a very good correlation in many criteria used for diagnosis in human melanoma and the B16F10.9 mouse model. (ii) We showed that in the absence of Ncr1, tumors of the same developmental stage and size feature more aggressive properties, which are correlated with worst prognosis and metastasis.

Conclusions: Thus, we concluded that the Ncr1 is involved in the structural modulation of melanocytic tumors.

We plan to further investigate the mechanisms underlying these results *in vivo* and *in vitro*, using RCM, conventional histology and functional assays.

P0154

Expression of the NKC-encoded C-type lectin-related glycoprotein Clr-a

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Purpose/Objective: The 'C-type lectin-related' (Clr) molecules (Clr-a to Clr-h) are C-type lectin-like receptors encoded by *Clec2* genes in the mouse Natural Killer Gene Complex (NKC). Except for *Clec2d* encoding for Clr-b, little is known about expression and function of members of the *Clec2* gene family. Interestingly, Clr-b is a ligand of the inhibitory NK cell receptor Nkrp1d with genes of Nkrp1d and other Nkrp1 receptors interspersed with *Clec2* genes in the centromeric region of the mouse NKC. To unravel the immunological significance of Clr molecules we are aiming at the characterization of expression and function of Clr molecules, including the orphan receptor Clr-a encoded by the *Clec2e* locus.

Materials and methods: Clr-a cDNA and mutants thereof were transfected in 293T and CHO transfectants. Clr-a expression was characterized by immunoblotting, immunoprecipitation and flow cytometry. Tissue-specific abundance of Clr-a transcripts was studied by quantitative RT-PCR (qPCR) analysis of various tissues of C57BL/6 and BALB/c mice. By immunizing rats with soluble Clr-a ectodomains, Clr-a specific mAb were raised.

Results: Tissue-specific expression of Clr-a was delineated from qPCR data and confirmed by immunohistochemistry. Low cell surface expression of Clr-a on transfectants (as compared to other Clr transfectants) was attributed to certain Clr-a domains by domain swapping experiments.

Conclusions: Cell surface expression of Clr-a is impaired due to sequence-intrinsic characteristics of Clr-a. Clr-a expression confined to specific epithelia suggests involvement of Clr-a in modes of tissue-specific immunosurveillance. Attempts to identify the (yet unknown) receptor for Clr-a are ongoing and will aid in testing this hypothesis.

P0155

Functional dissection of the hemi-ITAM of the human NK receptor NKp80

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Purpose/Objective: NKp80 is an activating NK cell receptor that binds to the genectically linked activation-induced C-type lectin (AICL) on myeloid cells. The NKp80-AICL interaction leads to mutual activation of NK cells and monocytes, and to cytolysis of malignant myeloid cells. Unlike other well-known activating receptors on NK cells, NKp80 seems not to associate with an accessory chain carrying an immunoreceptor tyrosine-based activation motif (ITAM), but signals via an hemi-ITAM like sequence motif in the cytoplasmic domain. In this study, we analysed effects of amino acid substitutions on phosphorylation of the hemi-ITAM and Syk recruitment by the hemi-ITAM as well as functional consequences thereof.

Materials and methods: NK-92MI cells were transduced with NKp80 and NKp80 mutants and resulting transductans used for biochemical und functional analyses.

Results: The tyrosine 7 residue (Y7) within the hemi-ITAM is phosphorylated upon stimulation of NKp80-transduced cells and is required for NKp80-mediated cytotoxicity. Replacement of Y7 by phenylalanine (Y7F) ablates phosphorylation of NKp80 and cytotoxicity upon triggering of NKp80. A peptide representing the phosphorylated hemi-ITAM-like sequence binds spleen tyrosine kinase (Syk) and Syk is phosphorylated upon cross-linking of NKp80, but not of the Y7F mutant. Phosphorylation and Syk recruitment is dependent on amino acids preceding tyrosine 7.

Conclusions: Taken together, we show that NK cell-activating signaling by NKp80 depends on the hemi-ITAM-like sequence and Syk, and that altered amino acids in the hemi-ITAM of NKp80 (as compared to the hemi-ITAM consensus) fine tune NKp80 phosphorylation and function as well as Syk recruitment.

P0156

Human alpha-1-antitrypsin transgenic mice exhibit reduced steady-state NK cell numbers, near lack of NK1.1$^+$ cells and intact responses to tumor cells

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Purpose/Objective: Alpha-1-antitrypsin (AAT) is being increasingly regarded as an immune tolerogenic protein; AAT prevents rejection of islet allografts in mice, diminishes inflammation and facilitates T

regulatory cell differentiation. At the same time, AAT protects tissues from injury and enhances animal survival during live bacterial infections. Lung-specific hAAT transgenic mice exhibit constitutive circulating levels of hAAT (<250 ng/ml), and are islet allograft-protective in the absence of exogenous hAAT treatment. Natural killer (NK) cells partake in transplant rejection both by direct killing of target cells and by inflammatory cytokine release. Thus far, the cellular targets of AAT have been identified as non-T cells, and include neutrophils, dendritic cells, macrophages and B cells; little is known regarding the effect of AAT on NK cells.

Materials and methods: Here, steady-state NK population size was determined in hAAT-transgenic mice. *In vivo* NK cell responses were evaluated by introduction of (syngeneic) B16 tumor cells and (allogeneic) NIH-3T3 cells to the peritoneal cavity.

Results: According to our results, total NK population size in the peripheral blood of hAAT transgenic mice was 47 ± 6.7% from that found in WT mice. Similarly, bone marrow NK cell population was 32 ± 11% of that found in WT mice. Notably, $NK1.1^+$ cells were nearly absent in the above mentioned tissues as well as in lungs and inguinal lymph nodes of transgenic mice compared to WT mice. B16 cells evoked comparable infiltrates of NK cells between the transgenic and WT strains, while NIH-3T3 cells evoked an infiltrate that was 43 ± 8.7% of that obtained in WT transplant recipient mice.

Conclusions: These results suggest that NK cells are targets of AAT activity. Further studies are required to identify the stage of NK cell lineage that is affected by AAT, and whether the protective activities of AAT might, in part, be NK cell-mediated.

P0157

IFN-g production by neonatal and adult blood $CD56^+$ NK cells and NK-like T cells in response to Trypanosoma cruzi and IL-15: differential kinetics and cellular subset responses

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Purpose/Objective: IFN-γ is a pro-inflammatory cytokine important for the control of infection by intracellular pathogens like *T. cruzi*, the protozoan parasite agent of Chagas disease. While it has been described for decades that $CD56^{bright}$ NK cells are the best early producers of IFN-γ, studies have recently shown that other subsets can be important like $CD56^{dim}CD16^+$ in a very early way, $CD56^{dim}CD16^-$ or NK-like $CD3^+$ $CD56^+$ cells. Since it is also known that cord blood cells produce less IFN-γ following pathogen contact, which has a role in the immune immaturity of the neonate, we investigated the differences in the early kinetic of IFN-γ production following *T. cruzi* and IL-15 stimulation and the cellular subsets responsible for this production in neonates and adults.

Materials and methods: Isolated cord blood and adult peripheral blood mononuclear cells were incubated with rhIL-15 (20 ng/ml) and/or live *T. cruzi* trypomastigotes in a 1:1 parasite-to-cell ratio for 2–48 h. After stimulation, cells were further processed in order to detect IFN-γ mRNA by quantitative RT-PCR, IFN-γ intracellular content by flow cytometry and IFN-γ levels in culture supernatants by ELISA.

Results: Our results show that whereas IFN-γ transcript levels rapidly increase during the first hours in response to *T. cruzi* associated with IL-15, the protein is not secreted before 12h and, globally, whatever the stimulus and the cell type, intracellular IFN-γ was not detected before 8–12 h of culture (though generally somewhat earlier in adult than in cord cells). After incubation with IL-15 and *T. cruzi*, $CD56^{bright}$ and $CD56^{dim}CD16^-$ NK cells were the best IFN-γ producers and this production peaked at 18h for adults and 24h for neonates. Besides, a low proportion of adult NK-like $CD56^+$ $CD3^+$ T cells also synthesized IFN-γ when parasites were combined with IL-15. Since, in adults, NK-like T cells constituted around 30% and $CD56^{bright}$ and $CD56^{dim}$ $CD16^-$ NK cells only 5 and 3% of $CD56^+$ cells respectively (versus respectively 1.5, 13 and 5% of $CD56^+$ cells in cord blood), their contribution to the global IFN-γ response is likely not negligible in relation to the size of their population.

Conclusions: These results indicate that the contribution of the various $CD56^+$ cell subsets to early IFN-γ production may vary in neonates and adults and these differences might contribute to the well-known immaturity of the immune system in early life.

P0158

Mapping the interaction site of the C-type lectin-like receptors KACL and NKp65

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Purpose/Objective: We previously reported the novel activating C-type lectin-like receptor (CTLR) NKp65. NKp65 is closely related to NKp80, an activating receptor expressed by human NK cells and a subpopulation of CD8 T cells. NKp65 was found to be a high-affinity receptor for KACL, a member of the human CLEC2 family of CTLR, encoded adjacently to NKp65 in the human Natural Killer Gene Complex (NKC). Strikingly, KACL expression is almost exclusively confined to human skin, i.e. keratinocytes. NKp65/KACL interaction has been shown to activate cytotoxicity and IFNg secretion of an NKp65- expressing NK cell line. From a delineation of the putative receptor binding site of NKp65 in the C-type lectin-like domain (CTLD) of KACL we expected further insights into the interaction mode of pairs of genetically linked C-type lectin-like receptors.

Materials and methods: Site-directed mutagenesis was employed to introduce mutations into the coding sequence of KACL. Mutated constructs were transfected into 293T and Cos7 cells and analysed by flow cytometry for KACL surface expression. Receptor binding was subsequently characterized using tetramerised ectodomains of NKp65 by flow cytometry.

Results: Based on the known crystal structure of the KACL-homologue CD69 (Natarjan et al, 2000; Llera et al, 2001), several amino acid residues exposed in the CTLD of KACL were selected for mutational analysis. KACL mutants were ectopically expressed on 293T or Cos7 cells and assayed for NKp65 binding. Certain amino acid substitutions in close proximity to the KACL C-terminus abolished NKp65 binding and thus likely define the NKp65 binding site.

Conclusions: Our results define KACL residues involved in the high-affinity interaction of KACL with NKp65 indicating that the membrane-distal KACL surface ligates NKp65.

P0159

Melanoma cells inhibit natural killer cell function by modulating their phenotype

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Purpose/Objective: Natural killer (NK) cells are a component of the innate immunity that play a fundamental role in cancer immune surveillance. Despite recent evidence suggests that NK cells can be considered promising effector cells in the adoptive immunotherapy of cancer, NK cell-based immunotherapy has resulted in limited clinical benefit. This may reflect the capacity of tumor cells to develop various escape mechanisms to avoid NK mediated killing. In this context, a number of cytokines, growth factors and enzymes synthesized by tu-

mor and/or stromal cells have been reported to exert suppressive effects on cells involved in immune response. In this study we analyzed the consequence of the *in vitro* interactions between melanoma cells and NK cells on NK cell phenotype and functional capability.
Materials and methods: Enriched NK cells were isolated from healthy donors using the Human NK Cell Enrichment Cocktail-RosetteSep and cultured for 6 days with irradiated melanoma cell lines in IL-2 (100 U/ml) at a Mel/NK ratio of 1:10. Specific inhibitors of the immunosuppressive factors IDO and PGE2 (1-methyl- DL-tryptophan, NS398) and the anti-IFN-γ-RI blocking mAb were added to the co-cultures. NK cells phenotype was analyzed on a FACSCalibur flow cytometer (BD Biosciences) and NK cells function was tested against melanoma cell lines in a ^{51}Cr release cytolitic assay.
Results: Our study shows that melanoma may interfere with NK cell function by down-regulating the surface expression of activating receptors including NKp30, NKp44 and NKG2D. Receptor modulation results in an impaired ability of NK cells to kill melanoma cells. This inhibitory effect is primarily mediated by indoelamine 2, 3-dioxygenase (IDO) and prostaglandin E2 (PGE2). In addition IFNγ-mediated signaling seems to play a role in the inhibitory effect of melanoma through the induction of IDO.
Conclusions: Our results help to better understand the molecular mechanisms responsible for the inhibition of NK cell function upon interaction with melanoma cells. In view of this immunosuppressive effect, new strategies might be developed to prevent inhibition of potentially efficient anti-tumor effector cells.

P0160
MHC-I modulation due to metabolic changes regulates tumor sensitivity to NK cells

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Purpose/Objective: Tumoral cells have a tendency, known as 'the Warburg effect', to use glycolysis to obtain energy instead of mitochondrial oxidative phosphorylation (OXPHOS). In a previous work we demonstrated that, at least in leukemic cells, this glycolytic phenotype correlated with loss of ERK5 expression and/or activity and, in consequence, with reduced MHC class I expression levels (Charni et al., J. Immunol. 185: 3498, 2010). Those results suggested that tumor cells could use this mechanism to evade immunosurveillance, since lower MHC-I expression should result in lower sensitivity to cytotoxic T lymphocyte (CTL) control. However, the same scenario can also result in increased sensitivity to NK cell-mediated cytotoxicity, as clearly demonstrated in leukemic cells in which ERK5 was eliminated by genetic means and MHC-I expression substantially reduced (Charni et al., J. Immunol. 182: 3398, 2009).
Materials and methods: In the present study, we have evaluated these outcomes, *in vivo* and *in vitro*, using the murine leukemia EL4 (or EG7) cell line and its derived EL4-ρordm; cell line, which lacks mitochondrial DNA. EL4-ρordm; cells should be considered as an extreme case of the Warburg effect, since they are completely dependent on glycolysis for survival and their MHC-I expression level is lower than that of parental EL4 or EG7 cells.
Results: While EL4-ρordm; cells were equally sensitive to cytotoxicity exerted by CTL than parental cells, they were more sensitive to activated NK cell mediated cytotoxicity exerted through the perforin/granzyme system. The increase in MHC-I expression induced by dichloroacetate (DCA) in EL4 cells by forcing mitochondrial respiration resulted in turn in a decrease in sensitivity to activated NK cell cytotoxicity. These results correlate with the reduced ability of EL4-ρordm; cells to generate tumors in syngeneic mice as compared with parental EL4 cells and have been confirmed in *in vivo* cytotoxicity assays.
Conclusions: Our present results show on one hand the importance of NK cells in immune antitumor surveillance, and on the other that the absence of OXPHOS does not seem to confer tumor cells an advantage in the context of immunosurveillance at least at the tumor establishment stage.

P0161
Natural killer cell education and tolerance under conditions of mixed haematopoietic chimaerism

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Purpose/Objective: *Introduction.* The missing self phenomenon explains that natural killer cells are 'educated' via inhibitory interactions with ligands, especially MHC-I, to tolerate self. The rheostat model states that this is a reversible and quantitative phenomenon, depending on the amount of signals a cell receives. Assuming NK cells integrate this input over multiple cell encounters, it may also depend on the ratio between cells with and without a critical MHC class I ligand, influencing their specific responsiveness. This clinically translates into chimaerism after haematopoietic transplantation. We aim to address whether education for NK cell responsiveness and missing self rejection is influenced quantitatively by the frequency of cells with a given MHC class I ligand.
Materials and methods: *Methods.* Mouse models: (i) The DL6 mouse, having mosaic expression of the introduced MHC class I (D^d) gene on a B6 background, where all cells are K^{b+} D^{b+}, while a while the proportion of D^{d+} versus D^{d-} cells varies among mice. (ii) Mixed hematopoietic chimaeras, with one donor carrying an extra D^d transgene, compared to the other donor, B6 (K^bD^b). In both models, the mice were challenged with a mixture of differentially fluorochrome labelled target cells of the following MHC phenotypes: $D^dK^bD^b$; D^bK^b; b_2m^-. Rejection was followed over time (in blood) and finally after sacrifice (in spleens).
Results: All DL6 mice were completely tolerant to $D^{d-}K^{b+}D^{b+}$ spleen cells, however, varying degrees of impaired missing self reactivity in the mosaic mice upon challenge b_2m^- cells, was observed. The relation between this missing self reactivity and the ratio of D^{d+} versus D^{d-} cells in the two mouse models is being investigated, using normal as well as tumor target cells. The *in vitro* responsiveness of NK cells in these mice will also be reported.
Conclusions: So far, our results suggest that complete tolerance develops robustly even towards ligand deficient host cells present only at low frequency under mosaic/chimeric conditions. One possible explanation is that the cells deficient in the additional MHC-I, modulate NK cell responsiveness. It remains to be investigated whether a frequency dependent quantitative education can be revealed when using tumor cells as targets.

P0162
Natural killer cells and NK receptors in granulomatosis with polyangiitis disease

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Purpose/Objective: GPA (former Wegener's disease) is a rare autoimmune disease characterized by severe necrotizing vasculitis of small size

vessels associated with granulomatosis. There are two main forms of GPA: a localized form characterized by granulomas mostly in lungs and upper airways and a generalized or diffuse form characterized by disseminated necrotizing vasculitis often involving the kidneys. We and others have identified a peculiar subset of CD4 T cells expressing some Natural Killer (NK) cells receptors in GPA patients. These cells are mainly distinguished by the aberrant presence of NKG2D, but they can express also other activating NK receptors (NKR) such as DNAM-1, 2B4 and some Killer Immunoglobulin-like Receptors (KIR). Moreover these CD4 T lymphocytes contain cytotoxic granules and show cytolytic abilities. These cells are present in granuloma and, as shown in our previous work (1), can contribute to vasculitis since they are able to kill endothelial cells in a TCR-independent way. Aim of our study was to characterize $CD4^+$ NK-like T cells and NK cells in patients with a localized or diffuse form of GPA, compared to patients with vasculitis without granulomas [Microscopic polyangiitis (MPA)] or with granulomatosis without vasculitis [Sarcoidosis (SAR)] to better understand the role of NKR and NKR-bearing cells in these pathologies.

Materials and methods: We used multiparametric flow cytometry and quantitative Real Time PCR to characterize lymphocytes phenotype and gene expression (NKR, signaling molecules, activation markers) from the different groups of patients and healthy controls.

Results: We evidenced major differences both in CD4 T cells and Natural Killer cells, especially in patients affected by a diffuse GPA disease, compared with healthy controls. Notably, we observed a higher expression of DAP12, NKG2D and KIR receptors on CD4 T cells and found that NK cells from the same patients displayed an early activated phenotype ($CD69^+$ NKp44-) and expressed more DNAM-1 receptor, compared with the other groups.

Conclusions: The presence of the activating adaptor protein DAP12 in T cells may represent an indicator of the acquisition of an aberrant and likely dangerous phenotype by CD4 T cells. A higher expression of this molecule, together with the aberrant expression of other NKR in CD4 T cells and the presence of activated NK cells, might participate in the pathophysiology of the diffuse vasculitis in GPA.

1. de Menthon et al. Arthritis Rheum. 2011;63(7):2116–26

P0163

Natural killer cells expressing the KIR2DS1 activating receptor efficiently kill T cell blasts and dendritic cells: implications in haploidentical HSCT

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Purpose/Objective: In the present study we investigate whether the expression of KIR2DS1 receptor by human NK cells may confer an advantage in their ability to kill allogeneic DC and T cell blasts.

Materials and methods: To this aim we generated NK cells clones from a C1/C1 Bw4/Bw4 $KIR2DS1^+$ healthy volunteer and we evaluated the effect of different NKG2A/KIR repertoire on the control of cytolitic activity against myelomonocitic dendritic cells (DCs) and T cell blasts derived from HLA-typed human peripheral blood mononuclear cells (PBMC) isolated from healthy volunteers.

Results: In allogeneic hematopoietic stem cell transplantation (HSCT), NK-cell alloreactivity is determined by the presence in the donor of NK cells expressing inhibitory KIR that recognize HLA class I allotypes present in the donor but lacking in the recipient. Dominant KIR ligands are the C1 and C2 epitopes of HLA-C. All HLA-C allotypes have either the C1 epitope, the ligand for KIR2DL2/L3, or the C2 epitope, the ligand for KIR2DL1/S1.

Here we show that, in alloreactive NK cell responses, KIR2DS1 expression represents a remarkable advantage as it allows efficient killing of C2/C2 or C1/C2 myelomonocitic dendritic cells (DC) and T cell blasts. When DC or T cell blasts were derived from C2/C2, Bw4/Bw4 donors, the activating signals delivered by KIR2DS1 could override the inhibition generated by NKG2A or KIR2DL2/L3 expressed on the same NK cell clone. Furthermore, substantial lysis of C2/C2, Bw4/Bw6 targets was mediated by $KIR2DS1^+$ NK cells co-expressing KIR3DL1. Importantly, in the case of C1/C2 targets, $KIR2DS1^+$ NK cells were inhibited by the co-expression of KIR2DL2/L3 but not of NKG2A.

Conclusions: Thus, KIR2DS1 expression in HSC donors may substantially increase the size of the alloreactive NK cell subset leading to an enhanced ability to limit GvHD and improve engrafment.

P0164

New prognostic markers in acute myeloid leukemia patients

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Purpose/Objective: To predict clinical outcomes in patients with Acute Myeloid Leukemia (AML) several characteristics as older age, poorer-risk cytogenetics, and performance status are commonly used. In the present work we have analyzed other prognostic markers in AML patients as NK cell phenotype and cytokine levels.

Materials and methods: PBMCs were obtained from 42 patients diagnosed of AML. NK cell ($CD3^-CD56^+$) surface analysis was carried out by flow cytometry. Plasma cytokine levels were measured in 42 AML patients, prior to initiation of therapy, and 58 healthy controls (HC). The concentrations of IL-1β, IL-2, IL-6, IFN-γ, IL-17A, IL-12p70, IL-8, IL-10, IL-4, IL-5 and TNF-α were analyzed using fluorescent bead-based technology. TGF-β was analyzed by ELISA technique. Patients were divided into two age groups, <65 and ≥65 years and compared with age-matched HC. Survival analyses were performed by the Kaplan-Meier method, and survival curves were compared using the log-rank test.

Results: Regarding NK cells activating receptors the univariate analysis showed that there are three factors associated with the increase of OS: age <65 year, good risk karyotype, and high expression of NKp46. The analysis of plasma cytokines levels showed that TNF-α, IL-6 and IL-10 levels were higher in AML patients from both groups of age. IL-8 was found increased in AML patients <65 years while the plasma concentration of IL-4, IL-5 and IL-12p70 was significantly higher only in AML patients ≥65 years compared to aged-matched HC. Kaplan-Meier analysis showed that low levels of IL-6 and high levels of IL-10 were associated with longer survival. Low IL-6 levels were associated with increased event-free survival.

Conclusions: Our results showed an aberrantly production of several cytokines in AML patients that may represent prognostic factors for disease response and survival in patients with AML. In addition, we show that higher expression of NKp46 on NK cells at diagnosis is associated with strong NK cell-mediated responses to leukemia and can be considered a novel prognostic marker for overall survival in AML patients.

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P0165

NKG2D is the main receptor involved in NK cell activation against NKG2D ligand-positive melanoma cell lines

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Purpose/Objective: The role of Natural Killer (NK) cells in immune surveillance against tumours has been recently underlined. Recent studies have demonstrated the expression of ligands for activating NK cell receptors in tumour cells from different histological origin. The facts that NK cells are able to respond rapidly and exert their cytotoxic function without previous sensitization to antigen place NK cells as a key component of the immune response against cancer. Melanoma cells can be recognized and killed by NK cells; however contradictory findings have been reported regarding the major activating receptors implicated in NK cell recognition.

Materials and methods: In order to analyze NK cell activation against melanoma cell lines we performed degranulation assays using IL-2-stimulated NK cells from healthy donors and melanoma cells with different expression of ligands for activating receptor. To test the participation of different activating receptors on NK cell degranulation, we performed blocking experiments by incubating NK cells with monoclonal antibodies against NKG2D, natural cytotoxicity receptors (NCRs) NKp30, NKp46 and NKp44, or DNAM-1 separately or together before co-culture with melanoma cells.

Results: We observed a variable NK cell degranulation against different melanoma cell lines. Blockade of NKG2D decreased NK cell degranulation in most melanoma cell lines analyzed. The higher degranulation of NK cells was observed against melanoma cell lines expressing high levels of NKG2D ligands showing a positive correlation between the percentage of CD107a/b$^+$ NK cells and the expression of ligands for NKG2D. We also observed a decrease in NK cell degranulation after blocking DNAM-1 or NCRs. These receptors were more involved in lysis of melanoma cells with low expression of NKG2D ligands.

Conclusions: Our results point to NKG2D as the most important NK cell activating receptor involved in the NK cell response against melanoma. Additionally, the cooperation of DNAM-1 and NCRs in this process has been shown to be complementary.

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P0166

Orchestration of NK cell phenotype and function by breast cancer-derived factors

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Purpose/Objective: NK cells are key components of protective anti-tumour immunity, as they recognise tumour associated antigens and stress-induced ligands in an MHC class I-independent manner. However, recent studies comparing NK cells from cancer patients with those from healthy donors have reported differences in the phenotype and function of NK cells in patients with cancer which could manifest as an impaired anti-tumour immune capacity. The involvement of tumour-derived factors in driving these effects is unclear and the aim of this study is to determine whether breast cancer-derived factors can influence NK cell phenotype and function.

Materials and methods: NK cells were isolated from peripheral blood of healthy donors by density gradient separation of PBMCs and subsequent negative isolation using magnetic beads. NK cells were incubated with 50% v/v supernatant derived from MDA-MB-436 human breast cancer cells for 24 h in the presence or absence of IL-2 (100 IU/ml). The expression of the serine protease granzyme B, CD8, the activating receptors NKp46, CD94 and NKG2D, and the inhibitory receptor CD158e1 by CD56^{low}CD16$^+$, CD56^{high}CD16$^-$ and CD56^{high}CD16$^+$ NK cell subsets was determined by multicolour flow cytometry. To assess function, NK cells were co-incubated with either K562 cells or MDA-MB-436 cells for 3 h following activation by IL-2 in the presence or absence of tumour cell-derived supernatants, and target cell death analysed by flow cytometry.

Results: In the presence of IL-2, incubation of NK cells with MDA-MB-436-derived supernatants significantly reduced CD56 expression (by 10–20% depending on NK subset), and this effect was more pronounced when NK cells were subsequently co-incubated with MDA-MB-436 cells (45–60% reduction). Expression of the other antigens was also influenced, although this was highly subject-dependent. CD8 expression was stable under all conditions. Incubating NK cells in MDA-MB-436 supernatant resulted in a 25% decrease in cytotoxicity against K562 cells.

Conclusions: Breast cancer cell-derived factors influence NK cells in a pro-tumourogenic manner. However, the effects appear to be subject-dependent and further work clarifying these effects is required. Current work is screening the immunoregulatory properties of factors derived from other breast cancer cells lines and identifying the molecules involved.

P0167

Recognition of human cytomegalovirus by NK cells

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Purpose/Objective: NK cells play a critical role in the defence against viral infections. Activation of NK cells is tightly controlled by both inhibitory and activating receptors and modulated by cytokines released along the anti-viral response. On the other hand, NK cells express several TLRs able to boost NK cell functions upon activation. The present study was undertaken to investigate whether NK cells could directly recognize Human Cytomegalovirus (HCMV).

Materials and methods: Human NK cells were purified from peripheral blood by negative selection using immunomagnetic techniques. After a 24h coculture with HCMV (TB40/E strain), NK activation was monitored by measuring CD69 expression and IFNg secretion by flow cytometry and ELISA, respectively. NK cell cytotoxic activity was measured by the CD107a-mobilization assay.

Results: HCMV recognition by NK cells resulted in the expression of CD69, increased IL-12-induced IFNγ secretion and improved cytotoxic response to infected fibroblasts and autologous monocyte-derived dendritic cells. Indeed, HCMV-primed NK cells showed increased activity of NKp46, NKp30 and CD16 activating receptors as measured in P815-based redirected degranulation assays. HCMV-induced activation was prevented by the pre-treatment of NK cells with bafilomycin A1 and by the addition of IFNAR and TLR2 blocking mAb along the coculture. Thus, indicating that sensing of HCMV by NK cells required a functional endocytic pathway and involved the production of type I IFNs and TLR2 activation.

Conclusions: Direct recognition of HCMV by NK cells results in the priming of NK cell effector functions, and could likely contribute to the anti-viral response *in vivo*.

P0168

Regulation of NK cell maturation and function by the actin-regulatory proteins Coronin1a and Coronin1b

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Purpose/Objective: NK cells are large granular lymphocytes, which contribute as an early defense system to the clearance of viral infections and the elimination of transformed cells. Recent work has begun to appreciate an important role of actin cytoskeletal dynamics in NK cell activation and function. We here further explored the role of the actin cytoskeleton for NK cells by analyzing the role the actin-regulatory proteins Coronin1a (Coro1a) and Coronin1b (Coro1b) in NK cell differentiation and function.
Materials and methods: Utilizing a genetic system of Coro1a- and/or Coro1b-deficient mice our study followed a multidisciplinary approach, which involved biochemical, cell biological and immunological methods to analyze the impact of actin-regulatory coronin proteins on developmental and functional aspects of NK cells.
Results: The analysis of naïve peripheral NK cells from spleen and lung revealed clear developmental alterations of NK cells from coronin-deficient mice. The NK cell differentiation status, as defined by CD27 and CD11b surface expression, was significantly shifted to a more immature NK cell state in Coro1a-deficient and Coro1a/1b-double-deficient mice as compared to wild-type controls. As the absence of Coro1a and Coro1b may also influence actin-dependent cellular functions during NK cell activation, we next analyzed NK function upon contact with target cells. For these experiments we utilized IL-15-primed NK cells, which showed similar expression of maturation markers and displayed normal granule contents in all genotypes. Importantly, Coro1a deficient NK cells showed defects in cellular degranulation and exhibited reduced cytotoxicity, which was further exacerbated by the additional loss of Coro1b in Coro1a/1b double deficient NK cells. These defects in degranulation and cytotoxicity in Coro1a/1b deficient NK cells were associated with impaired granule polarization towards target cells in NK cell/target cell conjugates. Furthermore, activation-induced secretion of inflammatory cytokines and chemokines, such as IFNg and CCL5, was significantly reduced in Coro1a and Coro1a/1b-double deficient NK cells.
Conclusions: Together, our study emphasises the importance of actin-cytoskeletal regulators for NK cells by identifying novel roles of the actin-regulatory proteins Coro1a and Coro1b in the control of NK cell maturation and function.

P0169

Regulation of NK cell subset localization at site of tumor growth in a model of multiple myeloma

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Purpose/Objective: NK cells play a pivotal role in the defense against tumors, due to the ability to kill target cells and to instruct other immune cells through cytokine production. Multiple myeloma (MM) is a malignancy of terminally differentiated plasma cells that are predominantly localized in bone marrow (BM), the main site for NK cell development. Our aim was to characterize the influence of MM growth on NK cell homing/trafficking to the BM and how this could affect NK cell-based anti-tumor response.
Materials and methods: We used a model of syngeneic orthotopic MM in C57BL/KaLwRij mice established by i.v. injection of the 5TGM1 tumor cell line. Disease progression was quantified after mice sacrifice by FACS analysis of $IgG2b^+$ cells into BM and spleen. NK cell subsets were defined as $CD11b^{low}$, $CD11b^{high}KLRG1^-$ and $KLRG1^+$ NK cells. *In vivo* homing experiments were performed by transferring total splenocytes or purified splenic NK cells from healthy mice into control and tumor-bearing mice at 3 and 4 weeks of tumor growth.
Results: We first observed that MM growth was sensitive to NK cells *in vivo* as it was markedly accelerated in mice depleted of $NK1.1^+$ cells. In addition, we could show that NK cells underwent activation and degranulated *in vivo* at tumor sites, as shown by increase of CD69 expression and of splenic $CD107^+$ NK cells at 3 and 4 weeks following tumor cell injection. *Ex vivo* experiments showed that target cell lysis and NK cell degranulation capacity were maintained at 3 weeks, while decreased after 4 weeks. Although all subsets of NK cells started to decrease in BM after 3 weeks, $KLRG1^+$ NK cells were minimally affected. Conversely, all subsets were markedly reduced in BM and spleen after 4 weeks, when tumor growth progressed. This findings correlated with modulation of chemokine receptor expression by NK cells and with defective NK cell homing to BM in tumor-bearing mice as shown by adoptive transfer experiments.
Conclusions: Our results suggest that when MM growth is limited, selected subsets of NK cells are excluded from BM. This can be partly attributed to NK cell redistribution that correlated with a stable anti-tumor activity. Conversely, further disease progression led to a strong reduction of NK cells at the tumor sites, possibly affecting NK cell-mediated anti-tumor response.

P0170

Retuning of mature NK cells after change of MHC class I (MHC-I) environment

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Purpose/Objective: The ability of NK cells to reject cells lacking self MHC-I depends on an education process which confers functional competence to the individual NK cell. Education also skews the repertoire of NK subsets defined by Ly49R expression in an MHC-dependent way. This may be harnessed in immunotherapy settings, by transfer of NK or bone marrow cells (BMC) from a host whose MHC-I molecules educate them so that they have the potential to recognize and eliminate the recipients malignant cells. It is therefore important to study how the host educating environment influences developing or mature NK cells. We have studied this in hematopoietic reconstitution and in adoptive transfer of mature NK cells.
Materials and methods: We confronted cells from $MHC^{+/+}$ mice (D^bK^b, $D^bK^bD^d$) with an environment where MHC-I was missing by transferring either BMCs or mature, NK cells to irradiated $MHC^{-/-}$ mice. Missing self capacity was tested by challenging the mice with MHC-I-deficient target cells. Reconstitution of NK cells and phenotypic analyses of activating receptors and maturation markers as well as repertoire assessment based on inhibitory receptors was performed.
Results: NK cells maturing from transplanted BMCs showed missing self rejection capacity against $MHC^{-/-}$ spleen cells by 2 weeks after transfer to autologous hosts. In contrast, they appeared tolerant to $MHC^{-/-}$ spleen cells after transfer to a $MHC^{-/-}$ deficient environment. The subset repertoire based on the expression of inhibitory Ly49 receptors in the donor-derived NK cells showed skewing towards that

seen in the recipient already 2 weeks after transplantation. Mature NK cells lost their ability to reject MHC-I-deficient spleen cells when transferred to MHC$^{-/-}$ hosts, but retained this ability in the autologous transfer. However, when mice where challenged with MHC-I-deficient tumor cells the transferred NK cells were equally capable of rejecting the tumor cells regardless of the MHC environment.

Conclusions: These results show that developing as well as mature NK cells adapt to the MHC environment. This suggests that tuning to tolerate normal cells lacking critical MHC-I ligands may still allow NK cells to detect and efficiently eliminate tumor cells lacking the same ligands. This supports the notion that the outcome of NK cell education is quantitative and context-dependent.

P0172

Surface expression of 'activation-induced C-type lectin' (AICL) tags cytokine-stimulated human NK cells

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Purpose/Objective: The 'activation-induced C-type lectin' (AICL) is encoded in the Natural Killer Gene Complex (NKC) adjacent to its receptor, the activating NK cell receptor NKp80. AICL is expressed by myeloid cells and NKp80/AICL interaction was shown to promote the cross-talk between NK and myeloid cells. Unexpectedly, we recently observed also substantial AICL expression by human NK cell lines and subsequently investigated regulation of expression, cellular localization, and functional recognition of AICL by resting and activated human NK cells.

Materials and methods: AICL expression by NK cells was assessed using qRT-PCR, immunoblotting and flow cytometry. Confocal microscopy revealed subcellular localization of AICL in NK cells. Functional assays included cytokine and degranulation assays. Hybrids of AICL and KACL proteins, the latter being a close AICL relative, were generated to map domains involved in intracellular AICL retention in 293 transfectants.

Results: Resting human NK cells contain intracellular stores of AICL glycoproteins associated with the Golgi complex. As evident from mutational analyses, the C-type lectin-like ectodomain of AICL determines intracellular AICL retention. However, upon exposure of freshly isolated NK cells to inflammatory cytokines, AICL expression is upregulated resulting in a substantial and prolonged cell surface expression. AICL on cytokine-stimulated NK cells is functionally recognized by resting autologous NK cells triggering their effector functions in an NKp80-dependent manner.

Conclusions: Our results show that AICL glycoproteins predominantly localize in the Golgi complex. While resting NK cells are devoid of surface AICL, there is substantial AICL cell surface expression following exposure to inflammatory cytokines facilitating recognition by autologous NK cells via NKp80. Hence, cytokine-stimulated NK cells become tagged by AICL surface expression for recognition by bystander NK cells.

P0174

The role of Natural Killer cells in Multiple Sclerosis pathogenesis

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Purpose/Objective: Multiple sclerosis (MS) is an autoimmune disease characterized by inflammation, demyelination, axonal/neuronal loss and gliosis of the central nervous system (CNS). The onset of disease occurs as a clinically isolated syndrome (CIS) which affects spinal cord, brain and optic nerves in 85% of young adults with MS. About 87% of MS patients exhibit a relapsing-remitting (RR) course of disease, characterized by acute attacks followed by partial or full recovery occurring at variable intervals. It is very well-known that immune system plays an important role in the pathogenesis of MS and natural killer (NK) cells is thought to be in part responsible for RR or progressive nature of the disease. Although many studies indicate the role of T cells in MS, surveys about NK cells are limited. In this study, cell frequency and effector functions of NK cells in untreated RR-MS patients, RR-MS patients treated with disease-modifying drugs (DMDs) and patients with CIS were investigated and the results were compared with age and gender matched healthy subjects.

Materials and methods: Primarily, surface expression of CD3, CD4, CD8, CD19, CD16 and CD56, and proportions of NK cell subsets were detected in peripheral blood samples of patient groups mentioned above. Peripheral blood mononuclear cells obtained from blood samples were cultured in the presence and absence of hrIL-2, hrIL-12 and hrIL-4. Following 24 h cell culture, IFN-g, IL-10 and IL-22 contents of CD56^{bright}CD16^{-} and CD56^{dim}CD16^{+} NK cell subsets were measured by flow cytometry. NK cell cytotoxic activity was measured using erythromyeloblastoid leukemia cell line K562, and the frequencies of NK cell subsets were detected after 72 h of culture.

Results:

Compared to healthy subjects:

1. Increased IFN-g levels were investigated in CD3^{-}CD16^{+} CD56^{dim} NK subsets of CIS, SP-MS and RR-MS patients receiving DMDs.
2. IL-10 levels were significantly increased in CD56^{dim}CD16^{+} and CD56^{bright}CD16^{-} NK cell subsets of unstimulated (US) SP-MS.
3. Increased IL-22 levels were observed in CD56^{bright}CD16^{-} subsets of USSP-MS patient.

Conclusions: Our first line of results shows that NK cells may contribute to the pathogenesis of different MS groups; however more patients are needed to underline their roles.

P0175

The role of NK cell – dendritic cell interactions during BCG vaccination

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Purpose/Objective: Bovine tuberculosis (TB), caused by *Mycobacterium bovis*, is an infectious disease of increasing incidence in the UK. Attenuated *Mycobacterium bovis* Bacille Calmette Guerin (BCG) has been successfully used to vaccinate humans against TB since 1921, however currently there are no available vaccines for cattle. Vaccination of neonatal calves with BCG provides significant protection against bovine tuberculosis. The increased efficacy of BCG may be attributed to the increased numbers of circulating innate effector cells observed in neonatal calves, particularly NK cells. Production of IFNg by NK cells and interactions with dendritic cells (DC) are hypothesised to polarise a Th1 immune response, thus interactions between NK cells

and DCs may be pivotal in driving protective immunity to bovine TB. Further understanding of the immune mechanism whereby BCG exerts protective immunity could allow targets for improved vaccination to be identified, leading to enhanced vaccine efficacy.
Materials and methods: Neonatal calves (BCG vaccinated and non-vaccinated age matched controls) were assessed for the frequency, phenotype and function of NK cells. NK cells and DCs from vaccinated and control animals were co-cultured *in vitro* to determine the way in which these two cell populations interact.
Results: Preliminary data obtained thus far shows an increase in NK cell populations and altered functional characteristics following BCG vaccination.
Conclusions: Further work to elucidate the reciprocal interaction between NK cells and DCs is currently underway in order to determine the effects this has on the adaptive immune response following BCG vaccination of neonatal calves.

P0176

Type I interferon gene expression by non-mucosal mononuclear phagocytes requires poising by the commensal microbiota

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Purpose/Objective: Mononuclear phagocytes are an important component of an innate immune system perceived as a system ready to react upon encounter of pathogens. The role of the commensal microbiota for calibrating the activity of mononuclear phagocytes residing in non-mucosal lymphoid organs (e.g. spleen) is not well defined.
Materials and methods: Using mice that lack commensal microbiota, we have investigated the molecular program underlying the priming of NK cell responses by mononuclear phagocytes in the context of viral infections and in response to defined stimulation of Toll-like receptors.
Results: Here, we show that mononuclear phagocytes residing in non-mucosal lymphoid organs of germ-free mice fail to produce type I interferons (IFN-I) in response to microbial stimulation. Consequently, NK cell priming and anti-viral immunity are severely compromised. Pathogen-inducible expression of a set of inflammatory response genes, including the various IFN-I genes, require poising by signals from the commensal microbiota. While signaling downstream of various pattern recognition receptors and nuclear translocation of NF-κB p65 and IRF3 were normal in mononuclear phagocytes of germ-free mice, binding to their respective cytokine promoters was impaired, which correlated with the absence of activating histone marks such as trimethylated lysine 4 of histone protein 3 (H3K4me3).
Conclusions: Our data reveal a previously unrecognized role for postnatally colonizing microbiota in the introduction of chromatin level changes in the mononuclear phagocyte system, thereby poising expression of central inflammatory genes to initiate a powerful systemic immune response during viral infection.

Poster Session: Responding - Innate Signalling Systems

P0177
A novel mechanism of action in dendritic cells for a tick salivary immunomodulatory cystatin

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Purpose/Objective: Sialostatin L (SialoL), a cysteine protease inhibitor of the cystatin superfamily is present in the saliva of the hard tick *Ixodes scapularis* and it has the ability to inhibit dendritic cell activation. Previous work has shown that it negatively affects the production of TNF-α and IL-12 by DCs, and the expression of the costimulatory molecules CD80 and CD86. Our aim was to illuminate a possible mechanism of these immuno-modulatory effects of sialostatin L.
Materials and methods: We used spleen dendritic cells activated by LPS or IFN-β. SialoL was prepared in *E.coli*, LPS decontaminated. Activation of signalling molecules was determined by Western blot. Expression of IFN receptor on the cell surface was analyzed by flow cytometry. Gene expression of IRFs was examined by qPCR.
Results: Type I IFNs are critical for DC maturation. We show here that SialoL attenuates Signal Transducer and Activator of Transcription 1 (STAT-1) activation but does not affect activation of nuclear factor-κB (NF-κB) or mitogen-activated protein kinase (MAPK) p38 signalling pathways. STAT-1 activation is mediated by secreted IFN-β. By using recombinant IFN-β, we demonstrate that SialoL inhibited IFN β -triggered STAT-1 and STAT-2 activation. IFN receptor (comprising of two chains -IFNAR1 and IFNAR2) is upon ligation internalized and this influences the strength of signal which is transduced further downstream. The number of IFNAR1 on DC surface upon IFN ligation was decreased in the presence of SialoL (by 50%) suggesting that SialoL inhibits IFN signalling at receptor level, by enhancing internalization rate of IFNAR1.

IRF are transcription factors linked to IFN and playing important role in the maturation of dendritic cells. We found that expression of IRF-4 is up-regulated and IRF-7 is down-regulated in the presence of SialoL.
Conclusions: We present here two possible mechanisms that mediate the modulation of DC function in the presence of SialoL: i) through inhibiting interferon (IFN) signalling, and ii) by changing gene expression of interferon regulatory factors (IRF). Both mechanisms can account for the observed changes in maturation and cytokine secretion profile of dendritic cells upon their activation in the presence of SialoL.
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P0178
A role for connexin signalling in innate immunity

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Purpose/Objective: Staphylococcal cell wall components peptidoglycan (PGN) and lipoteiochoic acid (LTA) elicit a potent pro-inflammatory response in diverse cell types, including endothelial cells and keratinoyctes.Such pro-inflammatory mediators are also reported to modulate Connexin (Cx) expression in diverse tissues. We explored the impact of PGN and LTA isolated from *S. epidermidis* and PGN isolated from *S.aureus* on Cx43, Cx26 and IL-6 expression and hemichannel activity in mouse and human endothelial cell lines (b.End5 and E. A.hy926 respectively) and in HaCat cells, a human keratinocyte cell line.
Materials and methods: Hemichannel activity was monitored following acute challenge (15min) with BCWC by ATP release assays in the presence or absence of the hemichannel blocker carbonoxolene (CBX)(100 uM). mRNA was harvested following 6, 18 and 24 h challenge with relevant BCWC and Real-time-PCR analysis determined Cx43, Cx26, IL-6 and TLR2 expression levels. IL-6 expression levels were also monitored by ELISA.
Results: In endothelial cells PGN and LTA (0.1 μg/ml) from *S. epidermidis* induced TLR2 expression and elicited an IL-6 and TNFα response. By contrast in HaCat cells PGN (at concentrations up to 10 μg/ml) isolated from *S. epidermidis* had little effect on IL-6 or Cx expression expression yet that sourced from *S. aureus* inducedIL-6 and Cx26 expression with no impact on Cx43 ($n = 3$, $P < 0.005$).Acute challenge (15 min) of endothelial cells with PGN or LTA induced hemichannel activity as determined by increased ATP release that was blocked by co-incubation with a range of hemichannel blockers including CBX, Gap26 and $LnCl_3$. By contrast in HaCat cells only PGN [10 μg/ml] derived from *S.aureus* induced a hemichannel response ($n = 3$; $P < 0.005$). Inhibition of acute hemichannel activity prevented the later induction of IL-6 and TLR2 mRNA but had no effect on the induction of Cx43 expression in b.End5 cells. By contrast in HaCat cells, Cx43 expression was not affected by PGN [10 ug/ml] isolated from *S.aureus* but an increase in Cx26 expression was observed.
Conclusions: Our results reveal that acute activation of Cx hemichannel signaling bypro-inflammatory mediators such as PGN and LTA isolated from*Staphylococcusspecies* plays a key a role in the initiation of an early inflammatory response and that their impact on Cx expression and function is highly tissue specific.

P0179
Adenine methylation in conserved GATC DNA sequences modulates macrophage and dendritic cell activation

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Purpose/Objective: Pattern recognition is an important means for innate cells to sense microbial infections. Differences in the methylation status of microbial and mammalian DNA represent a key element to distinguish self from non-self. This is illustrated by unmethylated bacterial CpG sequences that trigger TLR9 and are currently employed as adjuvant for vaccines. In addition, several bacterial strain genomes contain conserved GATC motives in which adenine is methylated in position N6 (m6A). Whether these G(m6A) TC motives exert an immunomodulatory function and if so, which cells respond to these bacterial sequences is still unresolved. Here we aim to determine whether macrophages and dendritic cells (DCs) respond to bacterial G(m6A) TC sequences, and assess the functional outcome of this recognition.
Materials and methods: To this purpose, we transfected dsDNA sequences containing methylated or unmethylated GATC motives in murine macrophages and DCs, and compared their capacity to activate these cell types, influence cytokine production, and affect their functionality.
Results: We show that transfection of macrophages with methylated dsDNA results in higher expression of the activation markers CD69 and CD86 as compared to the unmethylated dsDNA, and led to higher production of IFNβ, as well as of other effector molecules. Similarly, methylated dsDNA induced higher CD86 expression in DCs. We are currently investigating by which mechanisms m6A motives induce such response, in particular whether antigen uptake, recognition and activation pathways are involved.

Conclusions: Activation with dsDNA sequences containing m6A motives results in increased expression of activation markers in macrophages and DCs. The functional consequences of these findings have yet to be determined. Our study will show whether G(m6A) TC motives are able to direct the immune response towards pathogens, and whether they could be employed as adjuvant to improve the efficiency of vaccines.

P0180
Crosstalk between the p38 and Akt pathways in macrophages

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Purpose/Objective: The AGC protein kinase Akt is activated downstream of PI3K signaling. Akt has well defined roles in cell survival and insulin signaling and recently has also been implicated in regulation of innate immunity. Akt is activated by phosphorylation on both Thr308 and Ser473. PDK1 phosphorylates Thr308 and although the identity of the Ser473-kinase has been harder to elucidate, the TORC2 complex (mTOR, rictor, mLST8, sin1 & protor) can phosphorylate Ser473 *in vivo*.

MK2 (MAPK-activated protein kinase 2) and MK3 are activated by p38α MAPK and play important roles in macrophages and dendritic cells. MK2 efficiently phosphorylates Akt *in vitro* on Ser473, and in some circumstances p38 inhibitors can block Akt activity in cells. While work on mTORC2 excludes a direct role for MK2 in Akt activation in most systems, the unexpected finding that in macrophages MK2 and 3 play a role in the TLR induced activation of RSK, another AGC kinase, led us to re-examine the possible role for MK2 in Akt activation in macrophages.

Materials and methods: Bone-marrow Derived Macrophages (BMDMs) were isolated from wild type, MK2/3 or rictor KO mice and differentiated *in vitro* before stimulation with 100 ng/ml LPS. Where indicated, cells were pre-incubated for 1 h with 5 μM p38 inhibitor SB203580, 1 μM mTOR inhibitor KU0063794 or 10 μM MK2 inhibitor PF3644022. Following stimulation, cells were either lysed then analysed by immunoblotting or lipids isolated and relative levels of PI(3, 4, 5) P3 measured using a TRFRET displacement assay.

Results: Akt is activated by phosphorylation on Thr308 and Ser473 in response to LPS in BMDMs, but this is blocked by the p38 inhibitor SB203580 or in MK2/3 DKO cells (Fig. 1A). The phosphorylation is unlikely to be direct as PI3K and mTOR (Fig. 1A) inhibitors can also block Akt activation in BMDMs. Furthermore, Ser473 was not phosphorylated in rictor knockout BMDMs (Fig. 1B).

Together this indicates that MK2/3 acts upstream of Akt. For activation, Akt must be first recruited to PIP3 in the membrane. We find that inhibition of MK2/3 activation blocks the accumulation of PIP3 following LPS stimulation (Table 1).

Table 1. Xxxxxx.

Treatment	PI(3, 4, 5)P3 fold stimulation (mean and SD $n = 6$)	PI(3, 4, 5)P3 fold stimulation (mean and SD $n = 4$)
Control	1.00	1.00
LPS 30′	5.50 ± 2.3	3.25 ± 1.2
LPS 30′ + SB203580	2.50 ± 1.1	1.30 ± 0.8
LPS 30′ + PF 003644022	2.73 ± 1.5	–

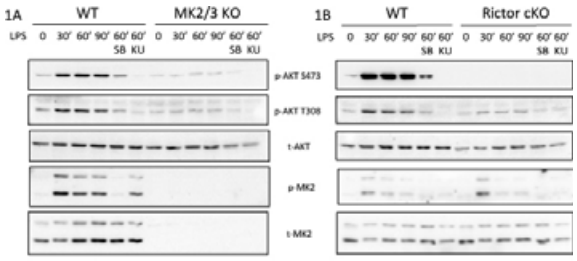

Figure 1

BMDMs were isolated from wild type, MK2/3 KO (A) or rictor KO (B) mice. Cells were stimulated with 100ng/ml for the indicated times in the presence or absence of 5μM p38 inhibitor SB203580 or 1μM mTOR inhibitor KU0063794. Cells were lysed and analysed by immunoblotting.

Conclusions: Our data reveals crosstalk between the p38/MK2 and Akt pathways in macrophages. MK2/3 do not appear to phosphorylate Akt directly, rather these kinases can modulate the localisation of Akt at the membrane, and hence its activation, by affecting PIP3 levels.

P0181
CYLD-deficiency protects against lethal infection with Listeria monocytogenes

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Purpose/Objective: CYLD is a deubiquitylating enzyme which plays a pivotal inhibitory role in immune responses. CYLD downregulates NF-kB activity by the proteolysis of K63-linked ubiquitin from IKKγ, TRAF2/6 and also negatively regulates the MAPK pathway by de-ubiquitinating TAK1.To gain insight into the function of CYLD in infectious diseases we studied the role of CYLD in listeriosis.

Materials and methods: C57BL/6 $CYLD^{-/-}$ and wildtype (WT) mice were infected (i.v) with various doses of *Listeria monocytogenes*. Colony forming units in the liver and spleen were estimated by plating them on BHI agar. Leukocyte infiltration in the liver was analysed by flow cytometry. Cytokine levels in serum were measured by cytometric bead assays. Regulation of NF-kB and MAPK pathways were analysed by WB.

Results: Upon infection with non-lethal doses of *L. monocytogenes* both WT and $CYLD^{-/-}$ mice survived the infection. However, upon infection with a lethal dose of the bacterium all WT mice succumbed up to day 7 post infection, whereas $CYLD^{-/-}$ mice survived indicating that CYLD inhibits protective host responses. The bacterial load in the liver of $CYLD^{-/-}$ mice was significantly reduced illustrating that the improved survival of $CYLD^{-/-}$ mice was partially due to a more efficient control of *Listeria* in the liver. In addition, recruitment of leukocytes including granulocytes, macrophages and CD8T cells, to the liver was significantly increased in $CYLD^{-/-}$ mice. This was accompanied by increased production of the pro-inflammatory cytokines IL-6 and IFN-γ in the liver of $CYLD^{-/-}$ mice. Additional WB analysis showed an increased NF-kB and MAPK activity in $CYLD^{-/-}$ mice as indicated by an increased phosphorylation of p65 and p38MAPK.

Conclusions: Based on these data, we conclude that enhanced activation of the NF-kB and MAPK pathways in $CYLD^{-/-}$ mice resulted in enhanced recruitment of leukocytes to the infected liver, an increased production of protective cytokine, which limited the spread of *Listeria* and enhanced survival of $CYLD^{-/-}$ mice.

P0182

Endothelial cells present an innate resistance to glucocorticoid treatment

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Purpose/Objective: In contrast to other chronic inflammatory diseases glucocorticoids (GCs) alone do not maintain sufficient remission in primary vasculitis. Reasons for this therapeutic failure remain unclear. We therefore investigated the molecular effects GCs exert on endothelial cells (ECs) and elucidated molecular pathways behind.
Materials and methods: We used a comparative approach treating human micro- and macrovascular ECs as well as monocytes long-term and short-term with GCs or GCs and TNF-a. Gene expression changes were analysed applying microarray technology, sophisticated bioinformatic work up and quantitative RT-PCR. Glucocorticoid receptor (GR) translocation processes were traced by cell fractionation assays and immunfluorescence microscopy.
Results: In ECs GCs completely fail to inhibit the expression of immune response genes both after sole GC exposure and after GC treatment of a TNF-a-induced proinflammatory response. In contrast, we see an impressive down-regulation of proinflammatory genes in monocytes. We verify that the GR is comparably expressed in ECs and monocytes and demonstrate nice translocation of ligand-bound GR allowing genomic GC actions. Refined gene expression analysis shows that in ECs transactivation causing GC side effects on growth and metabolism takes place whereas transrepression-mediated anti-inflammatory effects like in monocytes are missing. Insufficient induction of SAP30, an important constituent of the Sin3A-HDAC complex, in ECs suggests impairment of transrepression due to corepressor absence.
Conclusions: The impressive unresponsiveness of ECs to anti-inflammatory GC effects is associated with deficiencies downstream of GR translocation not affecting transactivation but transrepression. Our findings provide first molecular clues to the poor benefit of GC treatment in patients with primary vasculitis.

P0183

Expression, purification and biological characterisation of a novel type I interferon, IFN epsilon

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Purpose/Objective: The interferons (IFNs) are a large family of functionally and structurally related cytokines eliciting antiviral, antiproliferative and immunoregulatory functions. Our group discovered a novel gene while annotating the type I interferon locus with sequence similarity to other known type I interferons and named it IFN epsilon (IFNε). Unlike other IFNs, IFNε is constitutively expressed in the female reproductive tract and is not induced by pathogens. Furthermore, an *Ifne1* null mouse generated in our laboratory demonstrates an increased susceptibility to genital tract chlamydia and herpes virus infections. Little else is known about this cytokine due to a lack of commercial reagents. The objective of this work was to produce recombinant murine IFNε in order to characterise its biological activities and function.
Materials and methods: Recombinant mIFNε was produced in a baculovirus expression system and purified by immunoaffinity chromatography. IFNε's biological activities were characterised in antiviral, antiproliferative and immunoregulatory assays. The cytokine's ability to signal via the canonical type I interferon receptor and JAK-STAT signalling pathways were also investigated. *In vivo* activity was evaluated in a mouse model of genital tract Chlamydia infection.
Results: Recombinant mIFNε produced from a baculovirus expression system was shown to be >95% pure with endotoxin levels <0.1 EU/μg. IFNε demonstrated classical biological activities associated with type I interferons although at much reduced levels when compared to IFNα and IFNβ. Signalling studies demonstrated that IFNε signals through the heterodimeric type I interferon receptor complex and induced interferon regulated genes via JAK-STATs. Mice treated with mIFNε prior to genital chlamydia infection demonstrated lower levels of bacterial burden 3 days post infection.
Conclusions: This work outlines the expression, purification and biological characterisation of the novel type I interferon, mIFNε. Our results show that IFNε signals via the canonical type I interferon receptor to protect against genital tract chlamydia infection. These findings demonstrate a distinct role for this novel cytokine in maintaining homeostasis of the female reproductive tract.

P0184

Functional dissection of the TLR3, 7, 8, 9 interactome

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Purpose/Objective: The endosomal Toll-like receptors (TLRs) 3, 7, 8 and 9 recognize the nucleic acids of invading pathogens. These receptors are able to sense structurally distinct ligands and subsequently elicit an immune response fine-tuned to the respective threat. A set of ancillary proteins, which complement and modulate the different TLR molecular machineries would best explain this functional diversity. We thus aim to identify such cofactors of the endosomal TLRs.
Materials and methods: A mass-spectrometry based proteomics approach, involving tandem affinity purification of tagged endosomal TLRs, was performed to identify interactor candidates. Selected target proteins were stably knocked down in RAW 264.7 macrophages. After validation of the knock-down by qRT-PCR (quantitative real time polymerase chain reaction), these cell lines were systematically screened for their response to TLR agonists, using different cytokines (IL-6, IFNbeta) as read-out for inflammation.
Results: A proteomics approach to find interactors of endosomal TLRs identified several target proteins. Selected candidates were stably knocked down in RAW macrophages to assess their loss-of-function phenotype under stimulation conditions. Increased cytokine production was observed upon knock-down of two candidates, suggesting their involvement in negative regulation of endosomal TLR signaling pathways. These two candidates are currently subjected to in-depth validation, including FACS-based phagocytosis assays, gain of function analysis as well as intracellular localization studies. Finally, doxycyclin-inducible expression of tagged versions of the candidates in RAW264.7 cells will be established to be used to perform pull-downs on the interactor candidates followed by mass-spectrometric analysis. This will extend our knowledge on their proteomic environment, and, by combination with the initial TLR pull-down data, allow construction of a detailed and functionally annotated map of the TLR interactome.
Conclusions: Proteomics analysis led to identification of several interactor candidates of the endosomal TLRs. A role for two of these candidates in negative regulation of endosomal TLR signaling pathways is currently being validated.

P0185

Global innate immune response of human primary macrophages exposed to (1, 3)-beta-glucans

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Purpose/Objective: β-glucans and LPS are the immunostimulatory components of fungi and Gram-negative bacteria, respectively. β-glucans are known to activate the inflammasome and IL-1β secretion, however, the global innate immune response to β-glucan exposure has not been characterized. Here we have used a systems biology approach to study innate immune response activated by β-glucans and LPS in human primary macrophages.
Materials and methods: To characterize β-glucan induced secretomes in human macrophages the cells were stimulated with LPS, or β-glucans for 18 h, and the secreted proteins were analyzed by 4plex iTRAQ labelling combined with liquid chromatography-tandem mass spectrometry analysis. RNA microarray analysis was performed with Agilent technology to cells exposed to LPS and β-glucans for 6 h. Bioinformatic tools were utilised for data classification. Mouse bone marrow-derived dendritic cells from Dectin-1 deficient mouse and pharmacological inhibitors for SYK and Src tyrosine kinases were used in functional experiments. The PI3K inhibitor 3-methyladenine and small interfering RNAs of Beclin-1 were used for experiments to analyse the impact of autophagy in β-glucan induced secretome.
Results: We show that both β-glucan and LPS stimulation induced significant gene expression changes, but only β-glucans activated robust protein secretion through conventional and unconventional, vesicle-mediated protein secretion mechanisms, including exosome- and secretory lysosome-mediated release of proteins. The Dectin-1/Syk signaling pathway was essential for the release of IL-1b other unconventionally secreted proteins. β-glucan stimulation of human macrophages activated autophagy and our functional studies show that autophagy mediates unconventional protein secretion in β-glucan-stimulated human macrophages.
Conclusions: We provide the first comprehensive characterization of the secretome and associated intracellular signalling pathways involved in response to β-glucan exposure of macrophages. Our results demonstrate that activation of unconventional protein secretion was dependent on autophagy suggesting an important role for autophagy in innate immune response against fungal infections.

P0186

Heat stress facilitates innate anti-viral response through enhancing lipid raft-dependent induction of type I interferon

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Purpose/Objective: Host is usually subjected to various environmental heat stresses. However, effects of heat stress on innate immunity and the underlying mechanisms have not been clearly elucidated. We investigate the effects and mechanisms of heat stress on anti-viral response.
Materials and methods: The RNA viruses vesicular stomatitis virus (VSV) and Sendi virus (SeV), and the DNA virus HSV-1, were prepared and intraperitoneally injected (5×10^6 PFU) into mice. For *in vivo* heat stress, mice were anaesthetized and suspended in a water bath at 42¡C for 30 min. For *in vitro* heat stress of macrophages, dendritic cells (DC) and MEFs, cells were heat treated at 42¡C for 30 min in an air incubator containing 5% CO2. Virus replication in the liver and spleen was examined by both qPCR and titration assays. Activation of TBK1-IRF3 was evaluated by reporter assays and ELISA assays of IFN-α/β. Lipid rafts were isolated by sucrose density gradient centrifugation.
Results: We find that heat stress pretreatment of mice or macrophages/DC cells can inhibit virus replication and promote the production of IFN-α/β. However, heat stress can not significantly affect the anti-viral effects in $IFNAR^{-/-}$ mice or $IFNAR^{-/-}$ DC/macrophages. By using macrophages as infection model, we find that heat stress can promote both Toll-like receptor (TLR) 2, 4, 7, 9-triggered TBK1/IRAK1-IRF3/7 activation and IFN-α/β production. Moreover, heat stress pretreatment can enhance RIG-I-induced TBK1-IRF3 activation and IFN-α/β production. Based on our previous studies showing heat stress can mobilize recruitment of molecules into lipid raft, we further tested the effects of heat stress on lipid raft localization of TLR-associated molecules. We find that methyl-beta-cyclodextrin (cholesterol deprivation agent) can abrogate the effects of heat stress on anti-viral response in macrophages. Correspondingly, heat stress can enhance the recruitment of TLR2/4/7/9- and RIG-I-associated signaling components into Triton X-100 insoluble lipid rafts.
Conclusions: Our study suggests that heat stress positively regulates TLR-dependent and TLR-independent TBK1-IRF3 signaling pathway for initiation of type I interferon production and anti-viral response via lipid raft-dependent recruitment of TLR- and RIG-I-assocated signaling complexes.

P0187

IL-3-induced TAK1 activation controls the Erk1/2- and JNK2-dependent proliferation in mast cells

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Purpose/Objective: Stimulation of mast cells with IL-3 induces activation of Erk1/2, PKB/Akt, JNK1/2, p38 and finally leads to proliferation. Thereby the PI3Kδ/mTOR/p70S6K signalling pathway plays a predominant role.

Another important signalling pathway regulating mast cell effector functions is mediated by Myd88, Traf6 and TAK1. This signalling pathway typically leads to NFkB activation and finally to cytokine production. We investigated whether growth factors like IL-3 are also capable to activate mitogenic signalling pathways via TAK1.
Materials and methods: Cytokine release, proliferation and signaling pathways were assayed *in vitro* upon stimulation of bone marrow-derived mast cells (BMMCs) with IL-3. Several murine knockout strains and inhibitors were used to investigate the relevance of src-kinases, TAK1, the Erk- and the JNK-signalling pathway.
Results: Stimulation with IL-3 induced TAK1 activation mast cells (Kroeger et al., 2009). We found that the TAK1 inhibitor 5Z-7-oxozeaenol reduced the Erk1/2-, JNK1- and p70S6K- activation. Next we investigated whether Erk1/2 or JNK1/2 contributes to p70S6K activation and therefore to proliferation. The Erk1/2 inhibitor UO126 but not the JNK-inhibitor SP600125 reduced the IL-3-induced activation of p70S6K indicating that the IL-3-induced proliferation partly depends on TAK1 mediated Erk1/2 activation.

Interestingly, UO126, SP600125, but not 5Z-7-oxozeaenol blocked the IL-3-induced proliferation. This indicated that TAK1-inhibition induced a signaling pathway which compensates for the reduced activation of p70S6K, Erk1/2 and JNK1 after 5Z-7-oxozeaenol

treatment. Indeed we found that 5Z-7-oxozeaenol treatment increased the activation of JNK2.

Therefore we next analysed the role of JNK1 and JNK2 on the IL-3-induced signaling and proliferation. In *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ BMMCs the IL-3-induced signaling was not affected. Surprisingly, the IL-3-induced proliferation was reduced in *jnk2*$^{-/-}$- but not *jnk1*$^{-/-}$-BMMCs.

Conclusions: Our data show that the IL-3-induced proliferation is mediated by the TAK1-dependent Erk1/2- and the TAK1-independent JNK2-activation. Thereby activated TAK1 induces proliferation and simultaneously controls JNK2 activation to prevent uncontrolled mast cell proliferation.

P0188

Inflammasome activation in alveolar macrophages from healthy smokers

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Purpose/Objective: Recent studies have shown that a smoking increases the risk for autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. The mechanisms underlying the risk increase are not known, but we hypothesize that immunomodulation by tobacco smoke exposure could affect the systemic or organ specific immune response. Innate immunity, including inflammasome activation and TLR signaling are of particular interest since cytokines in these pathways are essential regulators of the immune system, including activation of the adaptive immune response.

Materials and methods: In order to investigate the innate immunity mechanisms related to smoking, samples were collected through bronchoalveolar lavage (BAL) from healthy smokers and non-smokers. mRNA expression of relevant genes and secretion of cytokines was measured in naïve and *in vitro* stimulated alveolar macrophages.

Results: Initial analyzes indicates that mRNA expression of the inflammasome component *NLRP3* as well as *pro-IL-1b* are upregulated in naïve macrophages from smokers, compared to non-smokers. In addition, cells from smokers show a tendency to increased IL-1§ secretion after *in vitro* stimulation with known NLRP3 inflammasome activators, compared to cells from non-smokers. In contrast, *TNF* expression is significantly downregulated in smokers, while other cytokines such as *IL-6* and *IL-8*, and the receptors *TLR2* and *4* are not differently expressed in healthy smokers and non-smokers.

Conclusions: Our results confirms that pro- and anti-inflammatory mechanisms are differentially regulated in alveolar macrophages from healthy smokers and non-smokers, and specifically indicate that tobacco smoke exposure might activate inflammasomes. Further research includes detailed studies of tobacco smoke induced signaling events and inflammasome activation, and its role in the regulation of systemic and lung-specific immune regulation.

P0189

JAKs mediate the actions of Interleukin-10 and IFNbeta feedback loops downstream of TLR4 in macrophages

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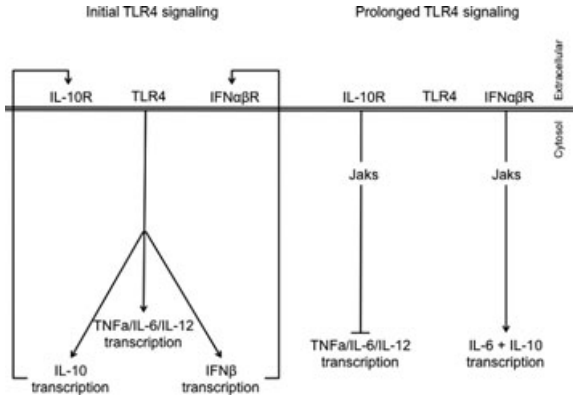
Purpose/Objective: The action of multiple cytokines is mediated by Jak/STAT signaling and Jak inhibitors have attracted considerable interest as potential drugs for autoimmune disorders.In macrophages, the response to the TLR4 agonist LPS can be modified by opposing IFN and IL-10 mediated feedback loops.As both these cytokines signal via Jak1/Tyk2, we investigated the effect of Jak inhibition on macrophages.

Materials and methods: To analyze the effects of Jak1/Tyk2 signaling in macrophages, we made use of the Jak inhibitor, Ruxolitinib, in combination with bone marrow derived macrophages from wild type, IFNabR or IL-10 knockout mice.A combination of qPCR, ELISA and immunoblotting were used to profile macrophage function in response to the appropriate stimuli.

Results: IL-10 is an important anti-inflammatory cytokine produced by macrophages that can act in an autocrine manner to inhibit the secretion of pro-inflammatory cytokines.Accordingly, in response to LPS, secretion of TNF, IL-6 and IL-12 was markedly increased in IL-10 deficient macrophages compared to wild type cells.IL-10 stimulates Jak1/Tyk2, and treatment of wild type cells with Ruxolitinib was found to increase TNF and IL-12 in response to LPS to a similar level to that seen in IL-10 knockout cells.Ruxolitinib also increased IL-6 production, but not to the level seen in IL-10 knockout cells.This could be explained by either an inhibition of IL-10 signaling or IL-10 production.We therefore looked at the effect of Ruxolitinib on IL-10 production. Ruxolitinib had no effect on the initial production of IL-10 but was required for its sustained production.Further work using IFNab receptor knockout mice demonstrated that this was due to a requirement for an IFNb feedback loop to sustain IL-10 transcription in response to LPS. Furthermore, IFNβ can induce IL-10 transcription, which was blocked by Ruxolitinib. IFNβ also promoted IL-6 transcription and, in line with this, the IFNabR knockout reduced LPS-induced IL-6 production.Thus IL-6 is subject to competing Jak dependent control mechanisms; its production is repressed by IL-10 but promoted by IFNβ.



Conclusions: Together, these results show that Ruxolitinib can alter cytokine production in macrophages by blocking IL-10 and IFNβ autocrine loops. These data also shows that IFNβ is sufficient to induce IL-10 transcription and is required for its sustained production in response to LPS.

P0190

Lipopolysacccharide signaling in human pericytes: potential role in inflammation and immune response

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Purpose/Objective: Activation of endothelium is a key event during the onset of inflammatory processes and implies the induction of chemokines, cytokines and adhesion molecules. Pericytes are embedded within the vascular basement membrane and play critical roles in vascular development and homeostasis; however, little is known about their role in inflammation. Our primary objective was thus to compare the response to lipopolysacccharide (LPS) in human endothelial cells and pericytes.

Materials and methods: To address this issue, we used cDNA microarrays-based analysis of gene expression of LPS-treated cells. We validated the microarray results using real time PCR. Supernatants were assayed for the production of secreted proteins with antibody arrays, and the expression of cell surface molecules was studied by flow cytometry. In addition, we explored the molecules implied in the signalling pathway, from the cell membrane to the nucleus.
Results: Incubation of human brain vascular pericytes with LPS for 4 h induced a significant increase in the transcription of 76 genes ($P < 0.05$), that were classified by functional annotation using gene ontology terms. Twenty-four terms in the list had a FDR value of <0.0001, including immune response, defense response and inflammatory response in the biological process category, and cytokine activity and chemokine activity in the molecular function category. LPS-treated pericytes released a plethora of cytokines and chemokines, as IL-6, IL-8, CXCL1 and CCL2; and upregulated cell surface expression of the adhesion molecules VCAM and ICAM. Furthermore, we assessed the expression of key elements of LPS signalling pathway (TLR-4, MD2, MyD88), as well as NF-KB translocation to the nucleus after LPS treatment.
Conclusions: LPS induces a robust modulation of gene expression in human pericytes that is similar to that induced in endothelial cells. These results indicate that pericytes contribute to the vascular cytokine and chemokine network and could play an important role in the inflammatory/innate immune response by influencing the site-specific recruitment of leukocyte subsets.

P0192
Src-family kinases and Syk are implicated in neutrophil extracellular traps formation

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Purpose/Objective: Upon activation, neutrophils release extracellular fibers or neutrophil extracellular traps (NETs), structures composed of chromatin and granule proteins that bind and kill pathogens. Accumulating evidence suggests that NETs can contribute to systemic and local pathologies such as sepsis, vasculitisand chronic lung pathologies like cystic fibrosis. Signal transduction mechanisms regulating NET formation are poorly understood. Given the important role of Src kinase family (SFKs) and Syk in inflammatory pathologies, we addressed whether these kinases regulated NET formation.
Materials and methods: NET formation was quantified by staining of extracellular DNA or visualized by fluorescence microscopy by standard procedures. As stimuli we used phorbol myristate acetate (PMA), beta-glucan (BG) and the *Pseudomonas aeruginosa* strain PAO1 (MOI 1).
Results: We found that beta-glucan, a ligand for the C-type lectin Dectin-1, is a strong inducer of NET formation. NET formation in response to beta-glucan requires both a SFK-dependent pathway and ROIs; in fact, in the presence of PP2 (inhibitor of SFKs) and DPI (an inhibitor of the NADPH oxidase) there is a reduction of about 50% in NET formation. Additionally, the new generation Syk inhibitor PRT-060318 reduces NET formation triggered by beta-glucan.

The most widely used activator of NET formation is the phorbol ester PMA that mimics the action of diacylglicerol an activator of protein kinase C (PKC). In presence of the Src family kinase inhibitor PP2 there is not a reduction of NET formation in response to PMA but there is a reduction in presence of DPI confirming previous studies.*P. aeruginosa* has been reported to induce NET formation. Notably, both PP2 and PRT-060318, effectively inhibited NET formation triggered by the strain PAO1 of *P. aeruginosa*.We also found that NET formation in response to beta-glucan and PAO-1 was defective in murine neutrophils deficient of the Src-family kinases Fgr and Hck.
Conclusions: These studies are instrumental to validate the use of Syk and SFKs inhibitors in pathologies, such as cystic fibrosis, COPD, sepsis and vasculitis, in which NET formation play an important role.

P0193
The Helicobacter pylori virulence factor VacA disrupts intracellular trafficking and blocks IFN-beta induced by *Lactobacillus acidophilus* in macrophages

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Purpose/Objective: It is indicated that an impaired immune response contributes to failed eradication of *Helicobacter pylori* after standard antibiotic therapy. Lactobacilli have been suggested to exert a preventative and therapeutic effect on gastritis caused by *Helicobacter pylori* infection. We aimed to identify the cellular and molecular pathways activated upon stimulation of murine bone marrow derived macrophages (BMDM) with *Lactobacillus acidophilus, Helicobacter pylori*, and both strains in combination.
Materials and methods: BMDM were stimulated with *Lactobacillus acidophilus, Helicobacter pylori*, and both strains in combination. Samples were analysed by microarray analysis, RT-PCR, ELISA and confocal microscopy.
Results: Microarray analysis revealed that *H. pylori* induces a high expression of neutrophil recruiting chemokines (Cxcl3, Cxcl1, Cxcl2) and cytokines (IL-1b and IL-1a). In contrast, *L. acidophilus* activates a strong Th1 response characterized by a high expression of IL-12 and IFN-β and the T-cell recruiting chemokines Cxcl10 and Cxcl11. Unexpectedly, when both bacteria are added in combination, *H. pylori* completely blocks the expression of IFN-β and IL-12 induced by *L. acidophilus*. Other strongly down-regulated genes are *Rgs1/2*, *Fgd2*, and *Dock8*, all involved in Rho, Rac and Cdc42 GTPase signalling and key regulators of the actin cytoskeleton. Two genes are strongly induced: *P2rx2* and *Pdxp,* both involved in actin cytoskeleton alterations.

We tested two of the main virulence factors of *H. pylori*: Cag pathogenicity island (cagPAI) and VacA (Vacuolating cytotoxin). The inhibitory capacity of the CagPAI mutant is identical to the WT, however, the VacA mutant is unable to block IFN-β. Phagosomal maturation inhibitors completely block the induction of IFN-β. Confocal microscopy revealed that instead of a quick endosomal release into the cytoplasm, *L. acidophilus* gets trapped in the endosomal/lysosomal compartment in *H. pylori* infected macrophages.
Conclusions: Our data indicates that *H. pylori* disrupts intracellular trafficking of *L. acidophilus* and thereby blocks IFN-β signaling via the virulence factor VacA and GTPases involved in actin cytoskeleton alterations. As *H. pylori* prevents the release of *L. acidophilus* from the endosomal/lysosomal compartment into the cytoplasm, other factors, *e.g.* cytosolic DNA sensors, possibly trigger the release of IFN-β in macrophages stimulated with *L. acidophilus*. Finally, even though *L. acidophilus* does not modulate the immune response induced by *H.*

pylori, we were able to identify possible novel therapeutic targets for treatment and prevention of gastric infections caused by *H. pylori*.

P0194
The mitochondrial protein, Toag-1, interferes with MyD88 dependent Toll-like receptor signaling in dendritic cells

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Purpose/Objective: We could recently show that Toag-1 is highly expressed in peripheral and graft infiltrating $CD4^+$ T cells and $CD11c^+$ cells DCs of tolerance developing recipients (Sawitzki *AJT* 2007) and the encoded protein is exclusively localized within mitochondria (Keeren *JI* 2009). Recently the importance of mitochondria in the regulation of host innate immune signaling, e.g. importance of TRAF6 translocation to mitochondria upon TLR1/2/4 ligand stimulation resulting in release of mROS, has been shown (Arnoult *EMBO* 2011). Here we investigated whether Toag-1 interferes with mitochondria-dependent events of TLR signaling in DCs and inhibits subsequent T cell priming.

Materials and methods: Bone marrow derived dendritic cells from C57BL/6 mice were transduced with an adenovirus encoding mouse Toag-1 or EGFP as a control. Upon stimulation with LPS, Pam3Cys or Poly(I:C) relative amounts of p-p38, MHCII, CD86, mitochondrial reactive oxygen species (mROS) and released cytokines were determined by FACS or Cytometric bead array. A transcriptional profiling was done by a mouse Toll-Like Receptor Signaling Pathway PCR Array. To determine their T cell priming capacities, stimulated DCs were co-cultured with eFluor450 labeled $CD4^+$ T cells from balb/c mice for 5 days.

Results: We observed an impaired LPS − and Pam3Cys-induced p38-MAPK activation upon Toag-1 overexpression in DCs. This was accompanied by a decreased release of inflammatory cytokines such as TNFa, IL-1a and IL-6, whereas upregulation of MHCII & expression of CD86 was not affected. Additionally we detected an abolished production of mROS. Using DCs from knock -out mice we could show that Toag-1 exclusively interferes with MyD88-dependent TLR signaling. Toag-1 overexpressing DCs display increased expression of SIGIRR, a negative regulator of Traf6-dependent signaling, but nearly completely abolished LPS-induced IL-2 expression. Subsequently, LPS and Pam3Cys stimulated Ad-Toag-1 transduced DCs failed to induce proliferation of and cytokine expression by allogeneic $CD4^+$ T cells.

Conclusions: Our data now show that Toag-1 regulates mitochondrial components of TLR signaling by preventing MyD88/ Traf6-dependent production of mROS and inflammatory cytokines but enhancing expression of negative regulators.

P0195
The role of triggering receptor expressed on myeloid cells (TREM)-1 and TREM-2 during influenza-induced inflammation

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Purpose/Objective: While Toll-like receptor (TLR) triggering by microbial products can lead to initiation of inflammation, the presence of other innate receptors is often required in order to amplify them. One such family of receptors is the triggering receptor expressed on myeloid cells (TREM) family. These membrane receptors are expressed on myeloid cells and associate with DNAX adaptor protein 12 (DAP12) in order to mediate inflammation. Different members of the family modulate inflammation in different ways. Upon association with TLRs, TREM-1 ligation induces signal transduction resulting in transcriptional activation of NFκB, thus amplifying the acute inflammatory response. Its role has been extensively described in bacterial-induced inflammation, such as sepsis, and a soluble form of TREM-1 (sTREM-1) is used as a diagnostic marker of both infectious and non-infectious inflammation. TREM-2, on the other hand, inhibits TLR responses and integrin-mediated migration, and is also involved in osteoclast differentiation and function.

Despite a clear role in infectious inflammation, to date, TREMs have only been studied in one type of viral infection, in which TREM-1 was found to be upregulated on neutrophils following filovirus infection. Exploration into the role of TREMs in other types of viral-induced inflammation is needed, in order to utilise knowledge of this recently discovered family of receptors for clinical benefit.

Materials and methods: We have utilised a murine model of influenza to investigate the expression of TREM-1 and TREM-2 on myeloid cell populations.

Results: Our studies have found increased surface expression of TREM-1 on myeloid cells during early time-points following influenza, while TREM-2 is associated with the resolution phase of inflammation. The role of TREM-1 and TREM-2 in the initiation and modulation of viral-induced inflammation in the lung will be discussed.

Conclusions: Expression of TREM-1 and TREM-2 is regulated during influenza-induced inflammation. Elucidating TLR amplifiers and regulators could provide novel drug targets for the treatment of inflammatory lung disease that carries a reduced risk of pathogen outgrowth.

P0196

TLR2 and MyD88 mediated signaling is essential for induction of inflammatory response against Brucella but not for replication of the bacterium inside human macrophages

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Purpose/Objective: Innate immune recognition constitutes an efficient mechanism against *Brucella*. However it is not clear how signals from different TLRs are orchestrated in leading to effective clearance of infection, especially in the human system. Our aim was to elucidate the involvement of TLR pathway members in the inflammatory response against *Brucella* and their effect on the outcome of *Brucella* infection in human macrophages.

Materials and methods: 293 cells, stable transfected with TLR2 or TLR4, were transiently transfected with NF-κB, AP-1 or CREB reporter driving the expression of luciferase gene for 16 h before stimulation. Differentiated THP-1 cells were transiently transfected with TLR2 or MyD88 siRNA for 48 h before stimulation with viable *Brucella melitensis* bacteria, at a multiplicity of infection (MOI) of 20. Luciferase activity was assayed with Dual-Luciferase Reporter System and cytokines in cell culture supernatants were measured by ELISA, 16 h after infection. Intracellular survival of *B. melitensis* was assessed with gentamycin protection assays, followed by lysis of THP-1 cells 0, 24, 48 and 72 h post infection.

Results: Viable *B. melitensis* bacteria activated NF-κB via TLR2 by eightfold but not via TLR4, in 293 cells. AP-1 or CREB activation was detected neither in TLR2 nor in TLR4 transfected 293 cells. Silencing of TLR2 and MyD88 genes resulted in the reduction of IL-6, TNF-α and IL-10 production in response to live *B. melitensis* bacteria by 50–55%, compared to control siRNA treated cells, whilst it had no effect on IL-1β production. Survival of *B. melitensis* in TLR2 and MyD88 silenced cells remained identical with the survival inside control cells, even 72 h post infection.

Conclusions: These results provide evidence that *Brucella* induces a TLR2-MyD88 dependent inflammatory response through NF-κB activation which in turn initiates transcription of pro- (TNF-α, IL-6) and anti- (IL-10) inflammatory cytokines. However, *Brucella's* survival inside human macrophages is independent of this response and of signals originated from TLR2 and MyD88. Synergy between intracellular innate immune receptors and TLRs to control *Brucella* infection in human system remains to be defined.

Poster Session: Responding — Leukocyte Cell Biology

P0197

Exploring neutrophil behaviour in a zebrafish model of inflammation through the generation of novel parameters using MatLab algorithms

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Purpose/Objective: Tracking of immune cells is key to understanding their behaviour during inflammation. Current software available for tracking of immune cells is limited. The aim of this study was to develop a MatLab package of segmentation and tracking algorithms to apply in the tracking of neutrophils, fluorescently labelled in our zebrafish model. The development of algorithms in MatLab allows us to explore parameters not available in other software packages such as directionality of neutrophil movement and neutrophil behaviour inside and outside of a wound region.
Materials and methods: Tail fin transection was performed on Tg(mpx:GFP) zebrafish (3 dpf) which were imaged on a spinning disk confocal from 1 h post injury (hpi) to 7 hpi. Images were exported from Volocity™ and analysed using MatLab m-files written for the tracking of immune cells. This is a fully automated analysis, after the user defines the initial thresholds based on fluorescent intensity of the images.
Results: Neutrophils from injured embryos had a lower meandering ratio and a greater speed than neutrophils tracked in uninjured embryos (meandering ratio 0.24 ± 0.03 versus 0.42 ± 0.05, $P = 0.003$; speed 4.03 ± 0.32 versus 1.31 ± 0.21 pixels/frame, $P < 0.0001$, $n = 3$), however there was no significant difference in total distance travelled (166.9 ± 17.4 versus 210.1 ± 30.28 pixels, $P > 0.05$). In injured embryos with a defined wound region, the oriented velocity towards the wound was 0.31 ± 0.24 pixels/frame. Once within the wound region, the oriented velocity of neutrophil tracks was -0.39 ± 0.32 pixels/frame; indicating that while the neutrophils travel at a similar speed, they are now travelling away from the wound. The 'in wound ratio' was 0.91 ± 0.04, indicating that once neutrophils enter the wound region they tend to stay, in the timeframe studied. The 'leave wound ratio' was 0.37 ± 0.03, a measure of the rate at which neutrophils move away from the site of injury once they have entered the wound region.
Conclusions: Using these algorithms, we can analyse the behaviour of immune cells in a more detailed way. In addition to previously available parameters such as meandering ratio and speed, more complex parameters such as velocity towards or away from a wound region and a measurement of how neutrophils behave while in a wound region are available. Combining this novel tracking technology with established assays in our laboratory will enable the further dissection of neutrophil fate following an inflammatory stimulus.

P0198

HIF-1 transcription complex is essential for translational control of myeloid cell functions

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Purpose/Objective: Myeloid hematopoietic cells are key effectors of host innate immune defence and are also majorly involved in autoimmune and hypersensitivity reactions. Upon malignant transformation myeloid cells also cause different types of acute myeloid leukaemia, which affect high numbers of people of different age groups worldwide. Normal and pathological responses of myeloid hematopoietic cells require adaptation to a signalling stress controlled by the hypoxia-inducible factor 1 (HIF-1) transcription complex. HIF-1 is known to trigger expression of genes responsible for glycolysis, angiogenesis and cell adhesion. However, since its functions in the biological responses of myeloid cell are not well characterised our aims were to elucidate the mechanisms of HIF-1-mediated regulation of myeloid cell functions.
Materials and methods: THP-1 human myeloid cells, LAD2 mast cells and purified primary human basophils were used to perform mechanistic studies. Western blot, ELISA, quantitative real-time PCR and biochemical assays were employed to conduct the study. The obtained results were validated using *in vivo* mouse models.
Results: Here we have shown for the very first time that in myeloid cells HIF-1 controls activation of mammalian target of rapamycin (mTOR) a key regulator of ribosomal translation of the proteins responsible for cell survival, proliferation and aging. HIF-1 was found responsible for control of the AMP/AMP-kinase branch of the signalling pathways regulating mTOR pro-translational activity. Moreover, we also demonstrated that mTOR is critically involved in HIF-1 activation through a biosynthetic mechanism.
Conclusions: The cross-talk of HIF-1 and mTOR pathways is responsible for the regulation of stress-adaptation mechanisms and translational control of myeloid cell functions.

P0199

Hsp70 is involved in different immune processes

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Purpose/Objective: HSP70 possess diverse functions that are essential for different intracellular processes and for protection of cells from adverse factors of microenvironment. HSP70 are intracellular proteins but there are evidences of cell surface and extracellular localization of HSP70. The functions of surface and extracellular HSP70 are still not clear. However it was shown that surface and extracellular HSP70 could activate different immunocompetent cells. These data indicate involvement of HSP70 in immune processes and pose the ground for study effects of these proteins in immune system.
Materials and methods: We carried out investigation of involvement of HSP70 in immune processes using different models and approaches.
Results: Our results showed that HSP70 was presented in lymphoid cell populations in intracellular, surface and extracellular forms. All these pools of HSP70 play essential role in lymphocyte populations. We revealed that intracellular HSP70 was involved in execution of programmed cell death and these proteins were translocated to the surface of apoptotic cells. Cytotoxic immune cells recognize and eliminate target cells bearing surface HSP70. It was also found that activated lymphoid cells produced extracellular HSP70. Different type of lymphocytes can bind and internalize exogenous HSP70. It is important that this internalization resulted in increased resistance of the cells to apoptosis. Another noteworthy property of extracellular HSP70 is their pronounced affinity to the surface of apoptotic cells and cells with denaturated membrane proteins. Along with effects mentioned above we revealed significant suppressing action of exogenous HSP70 on generation of reactive oxygen species (ROS) by phagocytes.
Conclusions: These results suggest an ability of HSP70 to restrain ROS production during inflammation with the purpose of prevention of the tissue from destruction. Further to the described data we discovered a distinct influence of stress hormones on HSP70 expression in immune cells that display an involvement of these proteins in neuroendocrine immunoregulation.

P0200
Inhibition of IL-27 attenuates atherosclerosis

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Purpose/Objective: Interleukin 27 binds to cytokine receptor WSX-1 to activate cells resulting in both pro- and anti inflammatory responses. In this study IL-27 was blocked *in vivo* to study the role in atherosclerosis.
Materials and methods: 239T cells were transfected with a lentivirus encoding the extracellular domain of WSX-1 with a human IgG tag or an empty virus. The medium was incubated with IL-27 prior to addition to activated splenocytes. Cytokine release was measured to confirm IL-27 blockade. LDLR $^{-/-}$ were injected i.m. with a sWSX-1 or an empty virus.A hIgG ELISA was performed to measure the release of sWSX-1. Mice with IgG levels higher than the control mice who received an empty virus were considered to be responders. The non-responders did not show an increased hIgG level in response to the treatment. Two weeks after the injection the mice were put on a high fat diet for 2 or 6 weeks to induce atherosclerosis. Lesion development, macrophage content and collagen content were determined in the aortic arch. FACS analysis was performed on blood, heart lymph node and the spleen to determine leukocyte activation. Splenocytes were also activated with αCD3 and αCD28 to determine cytokine production.
Results: *In vitro* the IL-27-mediated IFNγ and IL-10 secretion in spleen cells was reduced by the soluble receptor. *In vivo* blockade of IL-27 via the soluble receptor resulted in increased CD163 and IL-10 positive macrophages in the spleen after 2 weeks high fat diet. Neutralization of IL-27 via the soluble receptor resulted in a 31% reduction weeks in aortic lesion size after 6 weeks. The lesion size negatively correlated with the concentration of the soluble receptor in the serum of the responders.The collagen content was not affected, while there was a trend toward increased macrophage content. FACS analysis showed that blockade of IL-27 increased the circulating T helper cell population after 6 weeks high fat diet. Simultanously, the percentage of circulating regulatory T cells increased upon neutralization of IL-27. Surprisingly, no alterations were detected in the Th1 and Th17 populations. Furthermore, CCR2 expression on monocytes was reduced while CD163 expression increased.
Conclusions: Taken together these results indicate that blockade of interleukin 27 reduces lesion formation by inducing a less inflammatory immune response by inducing regulatory T cells and shifting monocyte differentiation towards anti inflammatory M2 macrophages.

P0202
Macrophage-delivered signals drive inflammation resolution in an *in vivo* zebrafish model

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Purpose/Objective: The inflammatory response is critical for maintaining tissue homeostasis. Neutrophils are the primary cell to respond to tissue injury or infection but can cause irreversible damage to surrounding tissues if they are not successfully removed. Neutrophils may leave inflammatory sites by death by apoptosis and subsequent uptake by macrophages or by migration away. Soluble mediators have been shown to be released from macrophages following ingestion of apoptotic cells *in vitro*, and these include prostaglandin E2 (PGE2). We therefore hypothesised that such mediators might be released *in vivo* and might drive neutrophil removal to aid successful resolution of inflammation.

Using zebrafish tail transection as a model of neutrophilic inflammation, we aimed to investigate the roles of macrophages on neutrophils during inflammation and to identify soluble mediators that can influence the outcome of inflammation resolution.
Materials and methods: Transgenic zebrafish allow leukocyte-specific ablation using Metronidazole to assess the role of macrophages during tail transection induced inflammation. Inhibitors and soluble mediators were added exogenously to dissect the molecular mechanisms involved. Neutrophil numbers, apoptosis and reverse migration of neutrophils were visualised *in vivo*.
Results: Tail transection in control larvae causes a peak number of neutrophils at 6 h post injury (hpi), which are fully resolved by 24hpi. Ablation of macrophages during inflammation causes resolution to fail, with significantly more neutrophils remaining at the site of tail transection 24 hpi. Neutrophil number at the site of injury in macrophage-depleted larvae is 14.41 ± 1.30 compared to 7.44 ± 1.14 in control larvae ($P < 0.001$). There is no significant difference in the number of apoptotic neutrophils however, at the site of injury.

PGE2 drives inflammation resolution by 12hpi in control larvae in a dose dependent manner.When PGE2 is added to macrophage-depleted larvae, neutrophil numbers are significantly reduced by 12hpi and maintained at 24hpi, with a mean of 8.894 ± 0.55 compared to macrophage-depleted larvae mean of 13.81 ± 0.70 ($P < 0.001$).
Conclusions: These data suggest PGE2 can drive inflammation resolution *in vivo*, and that macrophages produce soluble mediators that affect inflammation resolution, with PGE2 being a candidate compound suitable for further investigation.

P0203
Mechanisms of TGFß1 and IL-4 induced TREM-2 expression in monocytes

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Purpose/Objective: Triggering receptor expressed on myeloid cells (TREM)-2 suppresses the inflammatory response by reducing proinflammatory cytokines such as TNF-α and IL-6. TREM-2 expression is increased in chronic inflammatory conditions such as chronic obstructive pulmonary disease (COPD) and multiple sclerosis. Interestingly, most pro-inflammatory stimuli reduce TREM-2 expression. Therefore, the aim of this study was to identify inflammatory mediators that upregulate TREM-2 expression in human monocytes and investigate the mechanisms involved.
Materials and methods: Primary human monocytes or the human monocytic THP-1 cell line were utilised in this study. Human monocytes were isolated from peripheral blood of healthy volunteers using a Ficoll Paque Plus density gradient, followed by adherence. TREM-2 protein expression was measured by western blot analysis and immunofluorescence. TREM-2 mRNA expression was analysed by quantitative RT-PCR.
Results: Stimulation of THP-1 cells with either TGFβ1 or interleukin-4 (IL-4) increased TREM-2 protein expression. TGFβ1 and IL-4 also increased TREM-2 mRNA expression, six and twofold respectively. These results were also observed in primary human monocytes. TGFβ1-induced TREM-2 mRNA expression was suppressed by pre-incubation with the p38 inhibitor, SB203580. In contrast, inhibitors of PKC (bisindoylmaleamide), extracellular-signal-regulated kinase 1/2 (PD98059), phosphoinositide-3-kinase (LY294002) and the proteosome/nuclear factor kappa-B (MG132) had no significant effect on TGFβ1-induced TREM-2 mRNA expression.
Conclusions: The anti-inflammatory cytokines, TGFβ1 and IL-4 are involved in the resolution of inflammation and are upregulated in inflammatory conditions. TGFβ1 and IL-4 increased TREM-2 expression in monocytes. For TGFβ1, this was partially inhibited by p38

inhibition, suggesting that although p38 is involved, it is not the only mechanism. Current studies are analysing the role of SMADs in TGFβ1 induced TREM-2 expression and the mechanism of IL-4 induced TREM-2 upregulation. In conclusion, TGFβ1 and IL-4 may play a role in the increase in TREM-2 expression seen in chronic inflammatory conditions. These results support existing evidence for the role of TREM-2 in the resolution of inflammation.

P0204
Metal ions influence onto cytokines production by T lymphocytes in patients undergoing implantation therapy in dental medicine

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Purpose/Objective: The purpose of the study was to establish cytokines production under metal ions influence by T lymphocytes of patients in which individually the most appropriate material for implantation therapy and followed prosthetic treatment was choosen.
Materials and methods: Levels of twenty cytokines, typical for inflammatory reaction, were established in T lymphocyte cultures influenced by titanium and mercury ions obtained from 15 patients before implantation therapy (I), after implantation therapy and before prosthetic therapy (II) and after implantation and prosthetic therapy (III) using Quantibody human custom array.
Results: In non-stimulated culture significantly increased levels of IL-10, IL-11 and TNF-alpha between examinations II and III and significantly increased levels of ICAM-1, IL-10, IL-11, IL-15, TNF-alpha and TNF- beta between examinations I and III were found. After stimulation of lymphocytes by titanium significantly increased levels of IL-2, IL-10, IL-11, IL-13, IL-15 and MIP-1b between examinations I and III were found. In general, significantly increased concentrations of inflammatory reaction cytokines after stimulation by titanium as well as in the course of implantation therapy process were found.
Conclusions: This study confirmed the influence of metal ions onto levels of inflammatory reaction cytokines produced by lymphocytes of patients undergoing implantation therapy. The establishment of individually the most appropriate material for implantation therapy and followed prosthetic treatment should become common procedure.
Acknowledgement: The study was supported by research projects SVV 2012 nr. 264501 and PRVOUK-P28/LF1/6 of Ministry of Education, Youth and Sports, Czech Republic and by project NT 13087-3 of the Internal Grant Agency, Ministry of Health, Czech Republic.

P0205
Metal ions influence onto lymphocyte proliferation in patients undergoing implantation therapy in dental medicine

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Purpose/Objective: Purpose of the study: The purpose of the study was to prove the importance of metal intolerance diagnostics in patients undergoing implantation therapy in dentistry by determination of individually the most appropriate material for implantation therapy and followed prosthetic treatment.
Materials and methods: Materials and methods: Thirty-eight patients fullfilled the entrance criteria for implantation therapy, in these patients the test for metal intolerance (to Hg, Ti, Zr, Cr, Co) was performed three times [before implantation therapy (i), after implantation therapy and before prosthetic therapy (ii) and after implantation and prosthetic therapy (iii)] to choose the most appropriate material for implantation therapy and followed prosthetic treatment.
Results: Results: Based on results of metal intolerance test I to titanium, the patients were divided into two groups: Group A (24 patients with titanium implants-tolerating Ti) and Group B (14 patients with zirconia implants-with Ti intolerance). In Group A patients, prosthetic treatment was done by metaloceramics (Group A1-14 patients-tolerating Cr and Co) and by zirconia (Group A2-10 patients-with Cr and/or Co intolerance) based on metal intolerance test II. At least half a year after prosthetic treatment control metal intolerance test III was performed. In patients with Hg intolerance and/or galvanic currents in their oral cavity, amalgam fillings were replaced by non-metallic ones. Control metal intolerance test III showed the improvement of lymphocyte reaction in patients previously non-tolerating tested metals.
Conclusions: Conclusions: This study confirmed the importance of metal intolerance diagnostics in patients undergoing implantation therapy in dentistry. The establishment of individually the most appropriate material for implantation therapy and followed prosthetic treatment should become common procedure. Acknowledgement:

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P0206
Neuropilin-1 interferes with $CD4^+$ T cell responses

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Purpose/Objective: Naturally occurring $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells (Tregs) represent promising targets for the therapy of autoimmune diseases and allergy as well as cancer and certain infectious diseases. Our group identified the neuronal surface receptor Neuropilin-1 (Nrp1) to be highly expressed on murine Tregs, correlating with the expression of Foxp3 and *in vitro* suppressive function, thus representing a suitable surface marker for the identification of regulatory T cells. To characterize the role of Nrp1 in $CD4^+$ T cells we generated a transgenic mouse line overexpressing Nrp1 under control of the CD4 promoter (CD4-Nrp1).
Materials and methods: Lymphocyte distribution and frequencies as well as their Nrp1 expression were analyzed in wildtype (WT) and CD4-Nrp1 transgenic mice by flow cytometry. $CD4^+$ $CD25^-$ T cells were FACS-sorted, stimulated *in vitro* and their gene expression profile was assessed by Agilent gene chip analysis. The proliferative capacity of $CD4^+$ $CD25^-$ T cells from wildtype and transgenic mice was measured as loss of CFSE dye by FACS upon *in vitro* stimulation. In the contact hypersensitivity model, mice were sensitized with DNFB and ear-challenged on day 5. Ear swelling was evaluated as difference between challenged and unchallenged ear after 36 h.
Results: In CD4-Nrp1 transgenic mice we detected a significant increase in $CD4^+$ $Nrp1^+$ T cells, in particular in $CD4^+$ $CD25^-Nrp1^+$ T cells while the distribution of $CD4^+$ and $CD8^+$ T cells and $CD4^+$ $FoxP3^+$ Tregs in lymphoid tissues did not alter from wild type mice. $CD4^+$ $CD25^-$ T cells from CD4-Nrp1 transgenic mice exhibited lower proliferative capacity, expressed decreased levels of T cell activation markers and produced less proinflammatory cytokines than wild type cells upon stimulation *in vitro*. Microarray analyses of stimulated $CD4^+$ $CD25^-$ T cells from transgenic and wild type mice revealed differences in the gene expression profile particularly of molecules involved in T cell activation. *In vivo* studies using different models showed that Nrp1 expression on $CD4^+$ T cells also interferes with immune responses in this more complex situation.
Conclusions: Consequently, we hypothesize that Neuropilin-1 plays a role in the regulation of $CD4^+$ T cell responses.

P0207

Neuroregulator RET is critical to haematopoietic stem cell survival and function

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Purpose/Objective: Haematopoiesis is a developmental cascade that generates all blood cell lineages in physiological and transplantation conditions.This process relies on Haematopoietic Stem Cells (HSCs) capable to differentiate and to self renew.HSCs localise in microenvironmental niches that are thought to regulate their proliferation, survival and differentiation.However, the molecular mechanisms regulating HSC fate and homeostasis remain largely unknown.Here we show that the neurotrophic factor receptor RET is critical to HSC survival and function.
Materials and methods: This hypothesis was adressed taking advantage of cellular, molecular and genetic approaches both *in vitro* and *in vivo*, mainly multiparametric flow cytometer analysis and serial transplantation assays.
Results: We found that RET signalling molecules are expressed by HSCs and *Ret* ablation leads to reduced HSC numbers. Despite normal differentiation potential, RET null HSCs exhibit loss of *in vivo* stress response and reconstitution potential, being rapidly exhausted.RET deficient HSCs were unfit due to reduced *Bcl2* and *Bcl2l1*, downstream of impaired p38/MAP kinase and CREB activation.Accordingly, enforced expression of RET down-stream targets, *Bcl2 or Bcl2l1,* was sufficient to restore the *in vivo* activity of *Ret* null progenitors.Finally, activation of RET results in improved HSC survival and *in vivo* transplantation efficiency.
Conclusions: Thus, our work shows that RET is an essential cell-autonomous regulator of HSCs function, revealing neurotrophic factors as critical components of the HSC microenvironment.

P0209

New insights into the pro-inflammatory activities of Ang1 on neutrophils; induction of MIP-1ß synthesis and release

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Purpose/Objective: We reported the expression of angiopoietin receptor Tie2 on human neutrophils and the capacity of both angiopoietins (Ang1 and Ang2) to induce CD11b/CD18 activation and neutrophil migration. We also observed differential effects between Ang1 and Ang2 on neutrophils. Namely, Ang1, but not Ang2, does induce interleukin-8 (IL-8) synthesis and release, which prolongs neutrophil viability. Herein, we addressed whether Ang1 and/or Ang2 could modulate the synthesis and release of other inflammatory cytokines.
Materials and methods: Human neutrophils were isolated from venous blood of healthy volunteers.
Results: We observed by ELISA that Ang1, but not Ang2, mediates macrophage inflammatory protein-1 beta (MIP-1β; CCL4) synthesis and release by the neutrophils. Basal synthesis and release of MIP-1β was undetectable from 0 to 6 h post-isolation, whereas at 24-h, the basal synthesis and release was approximately 95 and 35 pg/million neutrophils, respectively. Treatment with Ang1 (up to 10 nM) and IL-8 (positive control; 25 nM) increased neutrophil MIP-1β synthesis by 310 and 307% and its release by 388 and 413% respectively. Neutrophils pretreatment with blocking anti-Tie2 antibodies did not inhibit the capacity of Ang1 to induce MIP-1β synthesis and release, suggesting that this effect of Ang1 is Tie2-independent and could rather be mediated upon integrins interactions. Pretreatment with p38 or p42/44MAPK inhibitors (SB203580 and U0126, respectively) abrogated Ang1 effects on MIP-1β synthesis and release. In addition, pretreatment with NF-κB inhibitors (Bay7085, Bay7082 and IKK inhibitor VII) also abrogated the capacity of Ang1 to induce MIP-1β synthesis and release. To assess the capacity of Ang1 to promote MIP-1β DNA to mRNA transcription and protein synthesis, neutrophils were pretreated with a transcription inhibitor (actinomycin D), and a protein synthesis inhibitor (cycloheximide). We observed that Ang1-induced MIP-1β synthesis and release was completely blocked by both inhibitors, suggesting that Ang1-induced MIP-1β synthesis and release is dependent on its DNA transcription and mRNA translation.
Conclusions: Our study is the first one to report Ang1 capacity to induce MIP-1β synthesis and release from the neutrophils, and that these effects are mediated through the activation of p38MAPK, p42/44MAPK and NF-κB signaling pathways.

P0210

Normal dendritic cell mobilization to lymph nodes under conditions of severe lymphatic hypoplasia

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Purpose/Objective: To address the requirement for lymphatic capillaries in the mobilization of dendritic cells (DCs) from skin to lymph nodes.
Materials and methods: We utilized mice bearing one allele of a functionally inactivated VEGFR3 where skin lymphatic capillaries are reported absent.
Results: Unexpectedly, DC mobilization from the back skin to draining lymph nodes was similar in magnitude and kinetics to control mice. By contrast, DC migration from body extremities, such as the ear and forepaws, was ablated. An evaluation in different regions of skin revealed rare patches of lymphatic capillaries only in body trunk areas where migration was intact. These regions had a local density of ~40% of control animals but were separated by vast regions of skin lacking lymphatic capillaries, such that there was a ~90% reduction in overall lymphatic density. Residual lymphatic capillaries expressed higher levels of CCL21 and collecting vessels of *Chy* mice had higher smooth muscle coverage, changes that likely aid in compensating for scarce lymphatic capillary density.
Conclusions: These studies identify anatomic differences in the impact of a mutant copy of VEGFR3 on lymphatic patterning and reveal that with regard to the regulation of DC migration, lymphatic capillaries are able to compensate for a marked loss in density to maintain normal mobilization of DCs from skin.

P0211

Packed red blood cells suppress myeloid dendritic cell and monocyte inflammatory responses in a whole blood model of transfusion

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Purpose/Objective: The basis for poor outcomes in some patients post transfusion remains largely unknown. Despite leukodepletion, there is still evidence of immunomodulatory effects of transfusion that require further study.In addition, there is evidence that the age of

blood components transfused significantly affects patient outcomes. Myeloid dendritic cell (DC) and monocyte immune function were studied utilising an *in vitro* whole blood model of transfusion.

Materials and methods: Freshly collected ('recipient') whole blood was cultured with ABO compatible leukodepleted PRBC at 25% blood-replacement-volume (6hrs). PRBC were assayed at [Day (D) 2, 14, 28 and 42 (date-of expiry)]. In parallel, LPS or Zymosan (Zy) were added to mimic infection.Recipients were maintained for the duration of the time course (2 recipients, 4 PRBC units, $n = 8$).Recipient DC and monocyte intracellular cytokines and chemokines (IL-6, IL-10, IL-12, TNF-α, IL-1α, IL-8, IP-10, MIP-1α, MIP-1β, MCP-1) were measured using flow cytometry.Changes in immune response were calculated by comparison to a parallel no transfusion control (Wilcoxin matched pairs). Influence of storage age was calculated using ANOVA.

Results: Significant suppression of DC and monocyte inflammatory responses were evident.DC and monocyte production of IL-1α was reduced following exposure to PRBC regardless of storage age ($P < 0.05$ at all time points). Storage independent PRBC mediated suppression of DC and monocyte IL-1α was also evident in cultures co-stimulated with Zy. In cultures co-stimulated with either LPS or Zy, significant suppression of DC and monocyte TNF-α and IL-6 was also evident. PRBC storage attenuated monocyte TNF-α production when co-cultured with LPS ($P < 0.01$ ANOVA). DC and monocyte production of MIP-1α was significantly reduced following exposure to PRBC (DC: $P < 0.05$ at D2, 28, 42; Monocyte $P < 0.05$ all time points). In cultures co-stimulated with LPS and zymosan, a similar suppression of MIP-1α production was also evident, and production of both DC and monocyte MIP-1β and IP-10 were also significantly reduced.

Conclusions: The complexity of the transfusion context was reflected in the whole blood approach utilised. Significant suppression of these key DC and monocyte immune responses may contribute to patient outcomes, such as increased risk of infection and longer hospital stay, following blood transfusion.

P0212
Production modi of IL-22

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Purpose/Objective: Psoriasis is a common disease and is generally regarded as a model disease for other chronic immune-mediated disorders like rheumatoid arthritis and Crohn's disease. The latest results from other and our lab strongly suggest that IL-22 plays a crucial role in the induction of the epidermal alterations in diseased skin of psoriasis patients. Currently, it is assumed that Th17-cells are the main producers of this cytokine in humans. By this study, we aimed to further investigate the production modes of IL-22.

Materials and methods: IL-22 production wasinvestigated in different human Th-subsets. Furthermore, we used SCID/beige, Rag1-, IL-2Rgamma-, CD1d-, IL-12p35-, and IL-23p19-deficient mice and psoriasis patient samples to confirm the *in vitro* results.

Results: First, we demonstrated that IL-22 can be produced both by T-cells following T-cell receptor (TCR) stimulation and by NKT-cells after activation with inflammatory cytokines with comparable levels. Interestingly, type-I interferons were able to potently inhibit these productions. Among different human Th-cell subsets, Th22- and Th1-cells produced high amounts of IL-22, whereas the secretion by Th17-cells was very low. The relevance of Th1-cells as an important source of IL-22 in psoriasis lesions was substantiated by significant positive correlation between levels of IL-22 and IFN-gamma, but not of IL-22 and IL-17A in samples of diseased skin from psoriasis patients. The presence of TNF-alpha, but not IL-6, was most critical for the generation of IL-22-producing Th22-cells. For IL-22 production after inflammatory cytokine stimulation IL-23, but not IL-12 played an important role. In line with this observation we found strong significant positive correlation between IL-22 and IL-23p19 levels in samples of diseased skin from psoriasis patients.

Conclusions: Taken together, our study suggests that in the psoriatic lesions both an antigen-specific activation via TCR and stimulation with inflammatory cytokines induced high IL-22 production and can explain the efficacy of anti-TNF-alpha- and anti-p40-therapy in psoriasis.

P0213
Reduced frequency of regulatory T cells in competitive athletes

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Purpose/Objective: The objective of our study was to evaluate whether the frequency of B and T lymphocytes and of natural killer cells in peripheral blood is influenced by moderate exercise in competitive athletes and in non-athletes, respectively.

Materials and methods: Blood samples from 11 competitive athletes and six non-athletes were obtained before, directly after, and 1 h after moderate exercise. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation and analysed by four-colour flow cytometry.

Results: Whereas the frequency distribution of B lymphocyte subpopulations ($CD19^+$ cells, class-switched/non class-switched memory cells, plasmablasts) was maintained in both groups, we found considerable differences in the T cell subpopulations:

While T cell subpopulations in the athletes group did not change compared to pre-exercise baseline, we found a significant induction of cytotoxic T cells (Tc; $CD3^+$ $CD8^+$; $P < 0.05$, compared to pre-exercise baseline), activated Tc ($CD3^+$ $CD8^+$ $CD69^+$; $P < 0.05$), and natural killer T cells (NKT; $CD3^+$ $CD56^+$; $P < 0.05$) in the non-athletes group directly after exercise, which returned to baseline within 1 h. In contrast, numbers of T helper cells (Th; $CD3^+$ $CD4^+$; $P < 0.01$) and regulatory T cells (Treg; $CD3^+$ $CD4^+$ $CD25^+$ CD127-; $P < 0.05$) were reduced 1 h after exercise in non-athletes.

Interestingly, even at baseline level Treg numbers in athletes were significantly reduced compared to non-athletes ($P < 0.001$).

The frequency of natural killer cells (NK; CD3-$CD16^+$ $CD56^+$ $Nkp46^+$; $P < 0.01$) increased in athletes and non-athletes after exercise. However, the increase in NK numbers was significantly alleviated in athletes compared to non-athletes ($P < 0.01$).

Conclusions: We found significant temporary changes in the frequency of Tc, Th, Treg, NKT, and NK cells in PBMCs from non-athletes, whereas cell frequencies in athletes, except for NK cells, did not change. Reduced Treg numbers in athletes imply a putative role of Treg cells in the immune regulation during chronic exercise and might contribute to the increased susceptibility to infections in competitive athletes.

P0215

Stimulation of dopamine receptor D3 expressed on $CD4^+$ T-cells favours Th1 immunity

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Purpose/Objective: Since inappropriate $CD4^+$ T-cell differentiation may lead to infection susceptibility, tumor progression or autoimmunity, this process must be tightly regulated. Lately, neurotransmitters have emerged as immune modulators as they may influence the function of immune system cells. Dopamine (DA) is a neurotransmitter that may trigger signals through five DA receptors (DARs) which often are coupled to modulation of adenylate cyclase activity. Considering that DAR D3 (D3R) is the highest affinity DAR and that selective D3R agonists favour secretion of IFN-γ by human T-cells *in vitro*, we aimed to determine the relevance of D3R expressed on $CD4^+$ T-cells as a modulator of adaptive immunity.
Materials and methods: We used a model of autoimmune colitis in which $CD45RB^{HIGH}$ $CD4^+$ T-cells from wild type (WT) or D3R knockout (D3RKO) mice were transferred into RAG1 knockout recipient mice. Disease severity was evaluated as weight loss. Th1, Th17 and Tregs cells infiltrated into lamina propia were analyzed by flow cytometry. Expression of D3R, IFN-γ and T-bet transcripts were analyzed by qPCR. *In vitro* T-cell activation was determined by IL-2 secretion and CFSE-assay. Differentiation toward Th1, Th17 and Tregs phenotypes was analyzed by intracellular staining of IFN-γ, IL-17 and Foxp3 in the $CD4^+$ or $CD4^+$ $CD25^+$ population respectively.
Results: Results show expression of D3R on both naïve and activated $CD4^+$ T-cells. Expression data was supported by signaling assays, as treatment with a selective D3R agonist reduced cAMP levels and ERK1/2 phosphorylation in WT but not in D3RKO $CD4^+$ T-cells. *In vitro* experiments show that polyclonally-stimulated T-cell activation was significantly impaired when $CD4^+$ T-cells lack D3R. In addition, *in vitro* polarization assays showed that the frequency of Th1, but not of Th17 cells, was notably reduced in absence of D3R. *In vivo* experiments show that D3RKO $CD4^+$ T-cells were unable to induce weight loss in recipient mice. In line with these results, a reduced proportion of Th1 cells was found in the colons of mice when D3RKO $CD4^+$ T-cells were transferred.
Conclusions: These findings reveal a novel modulatory mechanism of $CD4^+$ T-cell function, in which D3R-mediated signaling contributes significantly to efficient T-cell activation, to Th1 differentiation and to the progression of a $CD4^+$ T-cell-mediated autoimmune response *in vivo*.

P0216

Stimulation of Juvenile systemic lupus erythematosus blood cells induces IL-17 production

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Purpose/Objective: Th17 cells are a subset of $CD4^+$ cells known to produce the cytokine IL-17A. Th17 cells will differentiate from naïve T cells in the presence of IL-1β, IL-23 and IL-6 under the control of the transcription factor RORC. The pathogenesis of adult-onset systemic lupus erythematosus (SLE) has been linked to Th17 cells due to increased serum IL-17A [1]. There have been few investigations into the presence and role of Th17 cells within the more severe, juvenile-onset SLE (JSLE). The objective of this study was to investigate the mRNA and protein level of IL-17A and associated protein in JSLE patients and whether stimulation of JSLE peripheral blood mononuclear cells (PBMCs) induces IL-17 protein production.
Materials and methods: JSLE ($n = 5$) and control ($n = 5$) PBMCs were stimulated with CD3/CD28 activation beads at a 2:1 ratio for 2 days then cell-free supernatantsanalysed by IL-17 ELISA. Unstimulated PBMC RNA was extracted from JSLE ($n = 12$) and control ($n = 12$) patients. RT-PCR quantified levels of IL-17A, IL-23, IL-23R and RORC mRNA relative to RPL13A housekeeping gene. $CD4^+$ T cells were isolated from JSLE ($n = 16$) and control ($n = 10$) patients for IL-17A mRNA measurement. Plasma from JSLE ($n = 19$) and control ($n = 18$) were analysed for IL-17A levels by ELISA. The level of IL-17A in $CD4^+$ cells from JSLE ($n = 4$) and control ($n = 4$) was determined by Western blot.
Results: JSLE CD3/CD28 activated PBMCs produced higher levels of IL-17A protein compared to control PBMCs (383.9 pg/ml ±147.9 versus 55.2 pg/ml ±27.5). IL-17A and IL-23 mRNA expression was significantly higher in JSLE PBMCs compared to controls [IL-17A: 0.30 (±0.08) versus 0.07 (±0.02) $P = 0.02$; IL-23: 0.41 (±0.11) versus 0.34 (±0.25), $P = 0.04$]. RORC and IL-23R mRNA expression was also raised in JSLE compared to controls, although not statistically [IL-23R: 0.43 (±0.16) versus 0.16 (±0.07), $P = 0.19$; RORC: 0.52(±0.16) versus 0.3(±0.13), $P = 0.22$]. JSLE $CD4^+$ cellsexpressed higher levels of IL-17A mRNA compared to control $CD4^+$ cells (0.85 ± 0.14 versus 0.5 ± 0.13, $P = 0.06$). IL-17A was significantly higher in JSLE plasma compared to control (21.5 ± 5.2 pg/ml versus 7.2 ± 2.5 $P = 0.02$). An IL-17A band was detected in JSLE CD4 by western blot.
Conclusions: Th17 cells may be playing a pathogenic role in JSLE as JSLE PBMCs are able to secrete IL-17A upon stimulation and IL-17A is detected at significantly higher levels in JSLE plasma. The higher level of IL-17A associated proteins and transcription factor mRNA and IL-17A protein in JSLE patients indicates the presence of the pro-inflammatory environment required for Th17 differentiation.

1. Zhao, X. F., et al., Mol Biol Rep, 2010.

P0217

The role of CCR7 in cross-priming

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Purpose/Objective: The effective priming of T cell responses after encounter with various pathogens is important for pathogen clearance and the subsequent T cell memory induction. The contact of T cells and antigen-presenting cells (APCs), which is required for effective T cell priming, is a result of highly coordinated migration processes. CCR7 and its ligands are centrally involved in mediating interactions of naïve T cells with antigen-presenting dendritic cells (DCs) within secondary lymphoid organs (SLOs) during steady-state lymphocyte recirculation. In contrast, a role for CCR4 and CCR5 has been described under inflammatory conditions when mature DCs acquire the potential to immunogenically activate $CD8^+$ T cells: if DCs are activated by $CD4^+$ T_H cells in the presence of TLR-ligands like CpG, they start producing CCR5-ligands that selectively attract CCR5-expressing $CD8^+$ T cells for cross-priming (classical DC licensing). DCs can also get activated by NKT cells if glycolipid antigens like α-galactosylceramide (aGC) are present. In this case, CCL17 is produced by DCs, attracting CCR4-expressing $CD8^+$ T cells for cross-priming (NKT cell-mediated DC licensing). Here, we investigated the role of CCR7 relative to CCR5 and CCR4 in cross-priming under inflammatory conditions.
Materials and methods: We induced classical or NKT cell-mediated cross-priming, but excluded an effect of CCR7 by using $CCR7^{-/-}$ mice in an *in vivo* cytotoxicity assay.
Results: Despite the induction of CCR4 or CCR5 ligands, CCR7 and its ligands were not dispensable, as cytotoxicity in $CCR7^{-/-}$ mice was highly diminished. To account for the disrupted microarchitecture of SLOs in $CCR7^{-/-}$ mice, we transferred $CCR7^{-/-}$ OT-I (ovalbumin (OVA)-specific T cell receptor transgenic $CD8^+$ T cells) into WT mice.

After subsequent priming of recipient mice with OVA and CpG and/or aGC, levels of cytotoxicity were highly diminished, suggesting that CCR5 or CCR4 expression on T cells cannot compensate for CCR7 expression. Current efforts are aimed at clarifying whether $CCR7^{-/-}$ OT-I T cells cannot enter the white pulp or if CCR7 is needed within the white pulp.

Conclusions: CCR7 is necessary also under inflammatory conditions *in vivo* and cannot be replaced by upregulation of other chemokine receptors.

P0218
The Role of SPHKs and SIPRs in rheumatoid arthritis

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Purpose/Objective: Sphingosine kinase (SPHKs), SphK1 and SphK2, have been identified to phosphorylate sphingosine into sphingosine-1-phosphate (S1P). They are involved in a wide variety of cellular responses. S1P acts via S1P Receptors, $S1PR_1$, $S1PR_2$, $S1PR_3$, $S1PR_4$ and $S1PR_5$, all of which can be bound and activated specifically by S1P. A defect either in S1P signalling or S1PRs has been associated with many pathologies. Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by high levels of proinflammatory cytokine production. Elevated SPHK1, S1P, and $S1P_1$ have been reported in RA synovium. S1P signalling via $S1P_1$ promotes synoviocyte proliferation, increases COX-2 expression and prostaglandin E_2 production. This study comprehensively evaluated expression of SPHK1/2 and S1PRs in RA patients compare to healthy controls (HC) and osteoarthritis (OA) in peripheral blood (PB) and synovial tissues, respectively.

Materials and methods: mRNA and protein expression of SPHK1/2 and SIPRs were examined in neutrophils, monocytes and T lymphocytes of peripheral blood of 10 HC and RA patients, who met the diagnostic criteria of 2010 ARC / EULAR by QPCR and FACS, respectively. Competitive ELISA assessed SIP in serum of RA patients with remission and relapse and HC. We also performed SPHK 1/2 and SIPRs immunohistochemistry in synovial tissue from 4 RA/ OA patients.

Results: S1P was three times high in RA than those observed in HC, also was statistically higher in RA patient with relapse than remission. Intracellular expression of hSPHK1 in RA patients, with opposed to HC, was up regulated 1.4-folds in monocytes and T- lymphocytes with significance expression in CD4T cells. $hS1P_1$ and $hS1P_3$ exhibited a similar expression were up-regulated in neutrophils, while, $hS1P_5$ was statistical high in T cells. In contrast, $hS1P_4$ was down regulated in all sorted cells particularly in CD4T cells. As opposed to OA synovial tissue, RA synovial tissues were strongly positive for hSPHK1 and $hS1P_{1,\ 3}$ expressions. Quantitative analysis showed, SPHK1 and $hS1P_3$ are expressed in lining, sub lining and vascular endothelial layer, while $hS1P_1$ expressed mainly in lining and sub lining layers of the RA synovial tissue compared with OA.

Conclusions: These results suggest that SPHKs/S1P and its S1PRs might play a role in RA pathogenesis. The clinical significance of S1P as a biomarker for disease activity deserves further attention.

P0219
Tunneling nanotubes as a novel mechanism of intercellular communication between immune cells

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Purpose/Objective: The efficiency of the immune response relies on carefully orchestrated intercellular communication. Recently, tunneling nanotubes (TNT) have emerged as a novel mechanism of cell-cell communication. These long membrane protrusions can establish cytoplasmic continuity between distant cells and enable the exchange of cellular components. TNT have been found to connect a wide range of immune cells like B cells, T cells, macrophages, mast cells, NK cells and dendritic cells. This has prompted the assumption that TNT mediate intercellular communication between immune cells. In the present study we have aimed to elucidate the molecular basis for TNT formation and to investigate whether TNT enable exchange of cellular components between dendritic cells (DC).

Materials and methods: TNT formation and function was studied in HeLa, HEK-293T and U-937 transfectants, immature and mature DC. TNT were visualized by confocal microscopy of live and fixed cells, TNT functionality was assessed by transfer of stained vesicles/organelles. Interacting proteins were identified by coimmunoprecipitation, mass spectrometry and western blot analysis. Plasma membrane recruitment of interacting proteins was assayed by performing either immunocytochemistry or FRAP experiments.

Results: We report that the transmembrane MHC class III protein LST1 induces the formation of TNT. LST1-induced nanotubes were functional and enabled vesicle transfer between cells. While LST1 overexpression clearly enhanced both TNT formation and nanotube length, depletion of LST1 inhibited endogenous TNT formation and significantly reduced vesicle transfer. We identified several LST1-interacting proteins, and confirmed six binding partners, which contribute to nanotube development. A systematic analysis of these interactions allows us to propose a mechanistic model for nanotube formation.

Conclusions: We propose that LST1 recruits key effector molecules of actin reorganization to the plasma membrane and promotes the assembly of the molecular machinery required for membrane protrusions. Recent insights into the regulation of TNT formation during dendritic cell maturation and nanotube-mediated protein transfer between these cells will be discussed.

P0220

Use of the human eosinophilic cell line EoL-1 to investigate the molecular mechanisms regulating cyclin-dependent kinase inhibitor drug-induced apoptosis

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Purpose/Objective: Eosinophils are important in host defence against parasitic infection but also contribute to the pathogenesis of allergic diseases such as asthma and allergic rhinitis. Cyclin-dependent kinase inhibitor (CDKi) drugs induce eosinophil apoptosis and promote the resolution of eosinophil-dominant inflammation (Duffin et al., FEBS Lett. 2009; 583:2540−6; Alessandri et al., PLoS One, 2011;6;e25683). However study of the molecular mechanisms regulating eosinophil function and apoptosis is often difficult partly because of their scarcity in peripheral blood of normal healthy volunteers and their relative intractability to standard molecular biological techniques. Therefore having readily available cells such as the eosinophilic leukemia cell line, EoL-1, as a human eosinophil surrogate cell is useful for the study of granulocyte function. Here we investigated the effect and mechanism of action of CDKi on EoL-1 cell apoptosis.

Materials and methods: EoL-1 cells were treated with the CDKi drugs AT7519, DRB or flavopiridol and apoptosis assessed morphologically using light microscopy and by flow-cytometric detection of annexin-V/propidium iodide staining. Furthermore, we examined the effect of the CDKi drugs on the expression of the key granulocyte survival protein Mcl-1 and the cleavage of caspase-3 using western blotting techniques.

Results: AT7519, DRB and flavopiridol induced apoptosis in EoL-1 cells in a concentration- and time-dependent manner and caused marked Mcl-l down-regulation. The CDKi drug-induced apoptosis was caspase-dependent since rapid caspase-3 cleavage was observed and apoptosis was abrogated by the pan-caspase inhibitor Q-VD-OPh.

Conclusions: CDKi drugs promote caspase-dependent EoL-1 cell apoptosis via down-regulation of Mcl-1. These findings indicate that EoL-1 cells provide a good model system to study the effects of CDKi drugs and suggest that they could be used to investigate new biologically active CDKi drugs.

Poster Session: Responding — Monocyte, Macrophage & DC Functions

P0221
12/15-lipoxygenase mediates GC-induced anti-inflammatory mechanisms in monocytes

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Purpose/Objective: Glucocorticoids (GC) are drugs of choice for the treatment of many chronic inflammatory diseases. Our previous studies have shown that GC treatment does not simply suppress monocyte functions but induces a distinct anti-inflammatory phenotype in these cells. Also the treatment of LPS-stimulated monocytes with GC leads to re-programming of the cells towards a specific population involved in resolution of inflammation. Microarray analysis has shown up-regulated expression of anti-inflammatory and pro-resolution 12/15-lipoxygenase (12/15-LOX) in GC- and LPS/GC-treated monocytes. The aim of our studies was to determine lipoxygenase-dependent effects in GC-treated monocytes.
Materials and methods: Bone marrow-derived monocytes were isolated from wild type (wt) C57BL/6 and 12/15-LOX$^{-/-}$ mice and stimulated with GC and/or LPS. Gene expression was analyzed using quantitative RT-PCR. Protein expression was examined by Western Blot, Flow Cytometry and CBA technology. Functional assays were performed to analyze monocyte migration, chemotaxis, phagocytosis, killing and oxidative burst.
Results: GC induced reduction of adhesion to the plastic surface in wt as well as 12/15-LOX$^{-/-}$ cells. However, this effect was less pronounced in cells lacking 12/15-LOX. Migration was enhanced in GC- and LPS/GC-treated monocytes isolated either from wt or 12/15-LOX$^{-/-}$ mice. In contrast GC induced resistance to apoptosis was much more pronounced in wt as in 12/15-LOX$^{-/-}$ cells. GC-treated monocytes from wt but not from 12/15-LOX$^{-/-}$ mice show reduced production of ROS. Phagocytosis of carboxylated-modified latex-beads is increased in GC-treated monocytes. However, this effect was much weaker in GC-treated monocytes isolated from 12/15-LOX$^{-/-}$ mice. In contrast, phagocytosis of latex beads mimicking foreign particles was not significantly altered in monocytes isolated from wt and 12/15-LOX$^{-/-}$ mice.
Conclusions: GC- and LPS/GC-treatment induced anti-inflammatory and pro-resolving monocytes show increased expression of 12/15-LOX. Our results indicate that inhibition of ROS production as well as apoptotic processes, migration and adhesion by GC-treated monocytes are at least partially mediated by 12/15-LOX.

Specific targeting of the 12/15-LOX pathway may be a promising strategy to block undesirable inflammation with fewer side effects.

P0222
A role for syntaxin 3 in dendritic cell secretion

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Purpose/Objective: Dendritic cells (DC) act as a link between innate and adaptive immunity with the ability to drive a range of T helper cell subsets. The DCs responsible for this differ primarily in the cytokines they secrete. Soluble-*N*-ethylmaleimide-sensitive-factor accessory-protein receptor (SNARE) family members are proteins that drive membrane fusion and have been shown to play a key role in cytokine release. Little is known about the specific mechanisms involved in cytokine secretion from DCs, therefore we examined the expression of these proteins in DCs.
Materials and methods: We first examined the cytokine and chemokine secretion in DCs following stimulation with a range of Toll-Like Receptor (TLR) ligands by ELISA. SNARE mRNA expression, following this stimulation with the panel, was then analysed using RT-qPCR. Subcellular location of candidate SNAREs for cytokine/chemokine secretion was also analysed using confocal microscopy.
Results: Stimulation with a panel of TLR ligands in DCs resulted in differential cytokine and chemokine secretion. We then examined the expression of SNARE proteins and demonstrated that Syntaxin 3 but not SNAP 23 or Vamp 8 was upregulated in TLR4-stimulated DCs. Expression of Syntaxin 3 mRNA was also up-regulated in TLR7-stimulated but not in TLR2-stimulated DCs. Further analysis using confocal microscopy revealed that in TLR4 and TLR7-stimulated DCs Syntaxin 3 translocated from the cytoplasmic region into the plasma membrane. This was not seen in TLR2-stimulated cells.
Conclusions: Our data shows for the first time the differential expression of SNARE proteins in DCs following activation of TLRs and suggests a role for Syntaxin 3 in secretion of immune mediators. Given the importance of cytokine secretion by DCs in the pathogenesis of inflammatory disease, SNARE proteins may represent therapeutic targets in such diseases.

P0223
Activated platelets upregulate monocyte IL-10 production *in vitro*

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Purpose/Objective: Platelets accumulate at sites of inflammation and modulate adaptive immune responses, e.g. CD4$^+$ T-cell proliferation, *in vitro*. Little is known about the effects of platelets on the function of innate immune cells.

We aimed to investigate the effect of activated platelets on monocyte IL-10 and TNF-α production.
Materials and methods: Mononuclear cells (MNCs) from healthy donors were stimulated with the self-antigen thyroglobulin (TG) or the foreign antigen lipopolysaccharide (LPS) and cultured in the absence or presence of activated autologous platelets. To assess the potential roles of CD40L and platelet factor 4 (PF4) in immunomodulation by platelets, neutralizing antibodies to CD40L or the PF4-receptor CXCR3 were included in some experiments. Supernatants were analysed for IL-10 and TNF-α after 18 h, and monocytes were analysed flow-cytometrically for IL-10 secretion after 18 h. Platelet/monocyte aggregates were detected as CD14/CD61-double positive events.
Results: Addition of activated platelets to MNCs stimulated with LPS or TG resulted in an increased production of IL-10 and decreased production of TNF-α. Blockade of CD40L slightly inhibited this effect ($P < 0.05$), while blockade of CXCR3 had no effect. Two subsets of monocytes secreting high and low amounts of IL-10, respectively, were observed. The presence of activated platelets increased the proportion of the high-secreting subset following stimulation with TG ($P < 0.001$), and increased the amount of IL-10 produced per cell within the low-secreting subset ($P < 0.01$). Platelet/monocyte aggregates were found irrespective of whether platelets had been added

in vitro, but their proportion was increased after addition of activated platelets to cultures stimulated with TG (3.9 versus 8.0%, $P < 0.01$). Of note, platelet-bearing monocytes showed significantly higher IL-10 secretion than monocytes without adherent plateles ($P < 0.05$).
Conclusions: The data suggest that activated platelets exert an immunoregulatory function by enhancing IL-10 production by monocytes. This effect was dependent on direct platelet-monocyte contact, slightly dependent on CD40L and independent of PF4/CXCR3 interaction.

P0224
Activating APCs for immunological tolerance

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Purpose/Objective: Successful priming of adaptive immune responses is crucially dependent on innate activation signals that convert resting antigen-presenting cells (APCs) into immunogenic ones. APCs expressing the relevant pattern recognition receptors can be directly activated by pathogen-associated molecular patterns (PAMPs) to become competent to prime T-cell responses. Alternatively, it has been suggested that APCs could be activated indirectly by proinflammatory mediators synthesized by PAMP-exposed cells. However, data obtained with $CD4^+$ T cells suggest that inflammatory signals often cannot substitute for direct pattern recognition in APC activation for the priming of T helper responses.
Materials and methods: To test whether the same is true for $CD8^+$ T cells, we studied cytotoxic T lymphocyte development in mixed chimeric mice where coexisting APCs can either present a model antigen or directly recognize a given PAMP, but not both. To examine T cell antigen encounter on indirectly-activated APCs in unmanipulated mice, we targeted antigen to DNGR-1/CLEC9A$^+$ (TLR7-) APCs and co-administered a TLR7 agonist.
Results: We show that indirectly-activated APCs promote antigen-specific proliferation of naïve $CD8^+$ T cells but fail to support their survival and effector differentiation. Indirectly-activated APCs exceed immature ones in silencing of T cells.
Conclusions: Thus, inflammation cannot substitute for direct pattern recognition in T-cell priming and instead may facilitate tolerance. These findings have important practical implications for vaccine design, indicating that adjuvants must be judiciously chosen to trigger the relevant pattern recognition receptors in APCs. Conversely, the findings open up new avenues for the prevention of autoimmunity by 'vaccination' in the context of non-cognate adjuvants.

P0225
Activation of the Rar-related orphan receptor a by H5N1 leads to subsequent NF-kB inhibition and suppression of inflammatory responses in monocytes

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Purpose/Objective: Infections of the human host by highly pathogenic avian influenza viruses (HPAIV) are characterized by development of a cytokine storm and systemic spreading of infection. We assumed monocytes as main producers of cytokines to be involved in the emergence of a cytokine burst.
Materials and methods: Human blood-derived monocytes infected with A/Thailand/1 (KAN-1)/2004 (H5N1) or low pathogenic human influenza virus A/PR8/34 (H1N1) were analyzed in a genome-wide microarray analysis. Results were confirmed by quantitative Real-time PCR experiments and Western Blot. ROR$\alpha^{sg/J}$ mice from Jackson Laboratories were bred in order to receive ROR$\alpha^{-/-}$ fetuses. Embryonic liver stem cells from ROR$\alpha^{-/-}$ fetuses and corresponding Wt fetuses were used to establish an immortalized ER-Hoxb8 monocytic cell line.
Results: Surprisingly, genome-wide microarray analysis showed a strongly reduced inflammatory response of human blood-derived monocytes infected with HPAIV H5N1 compared to monocytes infected with low pathogenic human H1N1. Transcription factor profiling revealed overexpression of the Rar-related orphan receptor alpha (RORα), a nuclear receptor known to suppress NF-κB signalling. Western Blot analysis confirmed a stronger activation and nuclear translocation of RORα in case of H5N1 infection compared to H1N1 infection in human blood-derived monocytes. In line with this, translocation of the NF-κB subunit p65 was clearly reduced during H5N1 infection. Infection of ER-Hoxb8 knockout and Wt monocytes enabled us to prove coherence between RORα activation and NF-κB inhibition. Additionally, we could show a biological relevance of RORα activation during H5N1 infection as we observed a significantly stronger inflammatory and antiviral response in RORα knockout monocytes compared to corresponding Wt cells.
Conclusions: In summary, we present a novel mechanism by which H5N1 escapes the first immune reponse. This mechanism contributes to the high pathogenicity of H5N1 by enabling the virus to spread systemically.

P0226
Agrin, key player for the erythroid development

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Purpose/Objective: Erythroblastic islands (EI) are specialized niches in which erythroid precursors proliferate, differentiate and enucleate. They are composed of a central macrophage surrounded by erythroblasts at various stages of differentiation. Several roles have been proposed for macrophages at the erythroblastic niches, acting as a 'nurse' cell providing erythropoietin, inhibition of apoptosis, and engulfment of nuclei extruded by mature red cells.

We have previously demonstrated that agrin – a critical regulator of neuromuscular synapses-is a novel, non-redundant player at the hematopoietic stem cell niche. In addition we have recently demonstrated agrin requirement for survival and maturation of monocytic cells. On the basis of the observed defects we asked whether another well-defined hematopoietic niche – the erythroblastic island – was impaired.
Materials and methods: Agrin expression was evaluated by FACS and confocal analysis.

Murine MSCs were isolated from the bone marrow of C57Bl/6 female mice.

Clusters composed of splenic macrophages and erythroblasts were isolated as already described (Iavarone A. et al, Nature 2004).
Results: Here we show that agrin controls multiple steps of erytrocyte maturation. Agrin-deficient mice had bone marrow and splenic red pulp failure and showed decreased frequency and number of Myeloid Erythroid Progenitor (MEP) with an impairment of erythrocytes and monocytic development. This defect is partially rescued by the

addition of agrin-producing mesenchimal stem cells, thus suggesting a critical role of this proteoglycan in the cross-talk between mesenchymal and erythroblastic island cellular components.
Conclusions: Agrin-deficient mice are indeed characterized by impaired erythropoiesis, which may be due to the inability of agrin-deficient macrophages to support formation of erythroblastic islands. However, our results from reconstituted criss-cross islands supported a dual role of agrin in this cell-cell interaction. Interestingly in wild type recipient mice reconstituted with agrin-deficient bone marrow cells plus agrin-expressing MSC erythropoiesis was partially restored, even though both erythroblasts and myeloid cells lack agrin.

P0227

Analysis of differential gene expression in IFNß$^+$ versus IFNß- plasmacytoid dendritic cells *ex vivo* reveals different functional subsets

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Purpose/Objective: Type I interferons (IFN) consist of multiple IFNαs and one single IFNβ and are crucial mediators of protective immune responses predominantly against viral infections. Initial binding of IFNβ to the type I IFN receptor (IFNAR) induces a positive auto- and paracrine feedback loop leading to the expression of IFN-stimulated genes. Our previous studies using a bicistronic IFNβ/YFP reporter-knockin mouse model (IFN$\beta^{mob/mob}$) revealed that IFNβ expression is restricted to a minor population of plasmacytoid dendritic cells (pDCs) after *in vivo* TLR9 stimulation with CpG. Here, we wanted to determine whether IFNβ-producing pDCs harbor a specialized gene expression profile.
Materials and methods: CpG1668 was injected into IFN$\beta^{mob/mob}$ or IFNAR$^{-/-}$xIFN$\beta^{mob/mob}$ mice. Microarray and qRT-PCR expression analyses on *ex vivo* isolated splenic IFNβ/YFP$^+$ versusIFNβ/YFP$^-$ pDCs were performed and accompanied by immunofluorescent microscopy.
Results: *In vivo*, pDCs are the major source of IFNβ in the spleen after TLR9 stimulation with CpG. Notably, IFNβ-producing pDCs comprised only 10% of all pDCs. These IFNβ/YFP$^+$ pDCs displayed a different localization within the spleen as compared to pDCs not producing IFNβ. A microarray based transcriptome analysis of *ex vivo* isolated IFNβ-producing versus non-producing pDCs revealed that more than 1500 genes were differentially expressed between these two populations. Selected genes were submitted to qRT-PCR analyses and found to be differentially expressed between IFNβ-producing and non-producing pDCs also in IFNAR$^{-/-}$ mice.
Conclusions: IFNβ expression defines a subpopulation of splenic pDCs, which is unique not only with regard to the expression of IFNβ and its response genes, but also differs from pDCs not producing IFNβ in the expression of other immune-relevant genes. Intriguingly, signaling via the IFNAR plays no essential role in the expression of selected genes involved in T and NK cell activation and migration. Taken together, the differential expression of genes involved in immune modulation contradicts the current hypothesis of IFNβ induction being a stochastic event and provides a molecular mechanism for e.g. the selective recruitment of IFNβ-producing pDCs into the T cell zone and the induction of inflammatory immune responses mediated by these specialized pDCs.

P0228

Association of the LILRA3 (ILT6) deletion with B-NHL and functional characterization as an activator of the cytotoxic immune response

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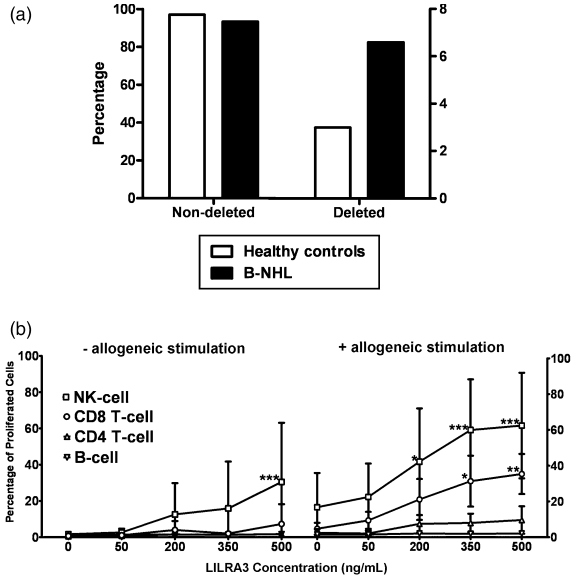
Purpose/Objective: B-non Hodgkin's lymphoma (B-NHL) is a malignant disorder which is predisposed in the impairment of the immune response. LILRA3 is the only member of the leukocyte receptor complex that exists as a solely soluble form, but is not functionally characterized. Deletion of LILRA3 has been associated with multiple sclerosis and Sjögren's syndrome, thus we are interested in studying whether the deletion of LILRA3 is also a risk factor for B-NHL and what function LILRA3 has in the immune response.
Materials and methods: For LILRA3 genotyping, we performed PCR on genomic DNA from 196 B-NHL patients and 798 healthy controls. For functional studies, recombinant LILRA3 was produced in a baculovirus system and PBMCs were obtained in heparinized tubes from blood bank donors. LILRA3 was conjugated to a fluorochrome to identify it binding subsets in PBMCs by flow cytometry. Using ^{3}H-thymidine assay, we determined whether LILRA3 induces proliferation in a mixed lymphocyte reactions (MLR), and resolved the specific proliferating populations using CFSE. A transwell MLR was performed to determine whether cell contact and/or soluble factors were crucial to the LILRA3-induced proliferation. We used cytometric bead array (CBA) as well as surface staining for HLA-DR, HLA-ABC, CD80 and CD86 on monocytes and B-cells to characterize the LILRA3-induced response on a molecular level.
Results: The homozygous LILRA3 deletion genotype is significantly overrepresented among B-NHL patients compared to healthy controls (Fig. 1A) ($P = 0.0325$; OR 2.273). Among the various PBMC subpopulations, LILRA3 bound specifically to monocytes, to a lesser extent to B-cells, but not to T- or NK-cells. LILRA3 greatly enhanced proliferation in an MLR, especially in CD8$^+$ T-cells and NK-cells (Fig. 1B). CD8$^+$ T-cells required cell contact to monocytes and allogeneic feeder cells to proliferate, whereas NK-cells required soluble factors produced by monocytes. Incubation of PBMCs with LILRA3 upreg-



ulated CD80 and HLA-ABC on monocytes, CD86 and HLA-DR on B-cells, as well as IL-6, IL-8, IL-1β in the supernatant.
Conclusions: LILRA3 deletion is associated with B-NHL. It is an activator of the immune response, via the activation of antigen presenting cells, which then induce the proliferation cytotoxic responder cells. This has interesting implications for viral immunity, since B-NHL is well known to be predisposed among HIV patients.

P0229
Autocrine BMP production regulates PD-L1 and PD-L2 expression in human monocyte-derived dendritic cells

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Purpose/Objective: Bone Morphogenetic Proteins (BMPs) are multifunctional secreted growth factors implicated in the regulation of differentiation, proliferation and cell death in numerous systems including the immune system. Previously, we described that the BMP signalling pathway is functional in monocyte-derived dendritic cells (MoDCs) which were found to express both the specific receptors and the Smad proteins required for the signal transduction. In the present study we investigated whether MoDCs are capable of producing BMP-4 and the role of this autocrine production during the maturation process.
Materials and methods: Immature DCs were generated from peripheral blood monocytes and maturation was induced by a proinflammatory cocktail. BMP signalling over the maturation was analysed by FACS and ELISA. The canonical signalling pathway during the maturation was inhibited by the addition of Dorsomorphin and the phenotypic and functional characteristics of the resulting mature DCs were determined by FACS, ELISA and q-PCR.
Results: We found that BMP-4 production by DCs was increased over the maturation process as well as the BMP signal transduction. When DCs were matured in the presence of Dorsomorphin the expression of the maturation markers PD-L1 and PD-L2 was especially reduced while cytokine production was not affected. As a result, these mature DCs presented an augmented ability to stimulate both T cells and NK cells. Eventually, the inhibition of BMP signalling during the maturation caused a reduced expression of IRF1, a transcription factor that positively regulates the expression of PD-L1 and PD-L2.
Conclusions: The present study demonstrates that the BMP signalling pathway regulates PD-L1 and PD-L2 expression in MoDCs during the maturation process through the IRF1 transcription factor. These findings also point out that the manipulation of BMP signalling might considerably improve the immunogenicity of MoDCs used in immunotherapy.

P0233
Cross species reactivity of Interleukin 34 (IL-34)

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Purpose/Objective: A second functional ligand for the CSF-1R, interleukin 34 (IL-34), has been identified in humans and mice. Together, CSF-1 and IL-34 are regulators of differentiation, proliferation, and survival of cells of the mononuclear phagocyte lineage. As yet, no distinct biological role of IL-34 has been discovered and no viable knockout mouse line has been reported. It has previously been reported that human IL-34 is considerably less active than the mouse protein in stimulating CSF-1R mediated mouse macrophage proliferation, a finding we have confirmed. We investigated the cross species reactivity of human and mouse IL-34 on the porcine CSF-1R by generation of a stable Ba/F3 cell line expressing full length functional porcine CSF-1R (pCSF-1R).
Materials and methods: An MTT bioassay using the Ba/F3 cells expressing pCSF-1R was optimised and performed with either rhIL-34 or rmIL-34.2 $\times$ 10^4 cells/well of a 96 well plate were plated and rmIL-34 or rhIL-34 was added. Cells were incubated for 48 h at 37°C. MTT solution (0.5 mg/ml) was added directly to each well and incubated at 37°C for 3 h prior to solubilisation overnight. Plates were read at 570 nm with reference wavelength of 405 nm.
Results: Both recombinant IL-34 proteins were almost equally active on the pCSF-1R expressing cells.We also compared the efficacy of human CSF-1 and IL-34 on the porcine CSF-1R, demonstrating similar activity. For both mouse and human IL-34 to be biologically active on the porcine CSF-1R, but no cross species reactivity, there must be a significant difference between mouse and human IL-34. Non-conserved contact amino acids between human and mouse IL-34 that may explain this, most notably, Asn187 (Lys mouse), which together with Leu186, Trp188 and Gln 189 makes up the α4 region required for biological activity. The change to Lys alters the charge from neutral to positive, and may produce a steric effect that influences the binding of IL-34 to CSF-1R, possibly by hindrance or repulsion.
Conclusions: This finding suggests that the pig could be an intermediate species in which to test therapeutic applications of recombinant IL-34 and combined with the successful expression of the porcine CSF-1R provides the tools for evaluation of IL-34 in pig and rodent preclinical models.

P0235
Dendritic cell trafficking is altered during hypertrophy-induced cardiac remodeling

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Purpose/Objective: Heart failure continues to present a substantial healthcare burden, despite recent treatment advances. The key pathophysiological process that ultimately leads to chronic heart failure is cardiac remodeling in response to chronic myocardial injury as for example arterial hypertension. Cells of the innate immune system could already be identified as important players in the induction of inflammation and tissue remodeling. Here, we investigated the role of dendritic cells (DC) on cardiac remodeling in a murine model of left ventricular hypertrophy.
Materials and methods: To induce cardiac remodeling, 12–14 week old female C57Bl6/N mice were subjected to transverse aortic constriction (TAC) and the relationship between DC subsets and left ventricular hypertrophy was studied. Innate immune cells were characterized and quantified by FACS analysis in the cardiac tissue of the left ventricle, the draining lymph nodes and the spleen to follow cellular migration and trafficking processes. DC migration was examined 3, 6 and 21 days after TAC intervention.
Results: At all three time points we observed a significant increase in the heart-weight/body-weight index in the mice after TAC compared to control animals. The percentage of $CD45^+$ cells in the tissue of the left ventricle increased from 8 to 14% at day 21 after TAC. The percentage of DCs in the myocardial tissue was significantly elevated at day 6 after surgical intervention. DC activation, as measured by CD86 expression, rose significantly at day 21. At the same time the percentage of DCs in the spleen was increased compared to days 3 and 6 after TAC and control mice.
Conclusion: Our current findings show an increased number of activated DCs in the myocardial tissue during cardiac remodelling

process. The altered number of DCs is caused by altered cell trafficking after myocardial injury. The functional relevance of DCs in the heart, as well as their migration, proliferation and cytokine pattern is currently under observation.

P0237

Detection of cytokines in human cell lines stimulated with metal ions

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Purpose/Objective: Human mucosal surface represents a vast area in which the external and internal environments come into contact. An important role in this contact is played by epithelial cells and the chemicals they produce. We therefore concentrated on studying the production of cytokines in cells stimulated with metal ions that are parts of dental alloys and whose corrosion products affect not only the oral cavity mucosa but, after ingestion, also the mucosal epithelium of the gastrointestinal tract.
Materials and methods: The study was performed on the macrophage cell line THP1 and two lines of human intestinal epithelial cells cultivated with Hg and Ni salts and also with other allergens such as Ti, Pd, Co and Cr. The cytokines were determined by the Ray Bio® Human Inflammation Antibody Array III, which permits the determination of 40 cytokines.
Results: The intestinal epithelial cell lines DLD 1 and HT 29 were found to respond differently to stimulation with metal ions. Cultivation with ions affected epithelial lines to various degrees of production of pro- as well as anti-inflammatory factors, growth factors and chemokines. Cultivation of the THP1 macrophage cell line with metal ions brought about a higher production of cytokines than that found with the epithelial cell lines; e. g. stimulation with chromium ions caused a significantly higher production of the pro-inflammatory factor Rantes. Nickel ions significantly increased the production of pro-inflammatory cytokine IL-6, chromium enhanced the production of IL-8. Cobalt activated the pro-inflammatory TNF beta.
Conclusions: The findings in the macrophage cell line THP1 were in keeping with the clinical experience implying the observation of high proliferative activity of mononuclear cells in patients diagnosed for undesirable response to dental alloys.

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P0238

Effects of interleukin-33 on phagocytosis and nitric oxide synthesis in macrophages

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Purpose/Objective: This study is aimed to enlighten effects of IL-33 on NO synthesis and the phagocytosis in macrophage 774.1 cell line.
Materials and methods: J774.1 cell line macrophages were used in this study. Cells were divided into several groups. In order to induce cells, low dose LPS (10 ng/ml), high dose LPS (1 μg/ml), IFN-γ (40 U/ml)$^+$ LPS (10 ng/ml), IL-33 in a variety of concentrations were applied.

In our study, NO production was measured by Griess reaction. Phagocytosis tests were done by using fluorometry. Kruskall-Wallis and Student's t statistical tests were applied by using SPSS.15.0 v. software.
Results: IL-33 was added into J774.1 cell cultures stimulated by IFN-γ plus LPS or high dose LPS alone and unstimulated. Then, nitrite levels were measured by Griess reaction. We observed that IL-33 in a variety of concentrations did not lead to NO production in J774.1 cell line. IL-33 also did not alter NO levels inJ774.1 cells stimulated by IFN-γ plus low dose LPS or high dose LPS alone in a dose dependent manner and in time-point experiments.

When added IL-33 into cultures, it caused to increasement in phagocytosis response of macrophages compared to negative controls ($P < 0.01$). Phagocytosis responses were found to be865% and 792% in response to IL-33 (20 ng/ml, 100 ng/ml, respectively). In cells stimulated by IFN-γ plus LPS, phagocytosis response was found to be 489%.
Conclusions: Macrophages are critical effectors and regulators of inflammation and the innate immune response. Although IL-33 did not lead to elevation in NO levels in macrophages, J774.1 cell line, it caused to a remarkable eightfold increase in phagocytosis response. In comparison with cells stimulated by IFN-γ and LPS, this phagocytosis response was twofold higher in cells induced by IL-33. It is a paradigm of macrophages that IFN-γ plus LPS induction leads to fully activation of macrophages. However, in our study we show that IL-33 is more potent effector for phagocytosis. Further experiments have been going on in order to elicit mechanisms for the effect of IL-33.

P0239

Efferocytosis by alveolar macrophages inhibit phagocytosis of *Streptococcus pneumoniae* by PG2/cAMP

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Purpose/Objective: Alveolar macrophages (AMs) are the first line of defense against microbial pathogens in the lung. The uptake of ACs by macrophages suppresses immune responses by releasing anti-inflammatory mediators such as TGF-β and PGE_2. The Scavenger receptor AI/II (SR-AI/II) and macrophage receptor with collagenous structure (MARCO) are the major receptors involved in the *Streptococcus pneumoniae* phagocytosis. However, the role of PGE_2 produced in response to efferocytosis in the phagocytosis of *Streptococcus pneumoniae* by alveolar macrophages is unclear.
Materials and methods: To induce early apoptosis, Jurkat cell were treated for 5 h with 8 μg/ml camptothecin (CPT). AMs were pre-incubated for 30 min pharmacological inhibitor and then incubated with AC (3:1) for 1 h, followed by FITC-labeled S. pneumoniae.
Results: We found that the pre-incubation for 1 h with AC (3:1) inhibited the ingestion of different *S. pneumoniae*-to-cell ratios by rat AMs (AMs:Sp 1:100 = ~ 43%, $^*P < 0.05$ versus AM^+Sp). The inhibition of *S. pneumoniae* phagocytosis by efferocytosis was partially reverted when endogenous PGE_2 production was inhibited with COX inhibitor indomethacin (AMs:Sp – 1:100 = ~ 25%, #, $P < 0.05$ versus AM^+Sp) and by adenylate cyclase inhibitor (AMs:Sp – 1:100 = ~ 17%, versus AM^+Sp). The suppressor effect of efferocytosis by AMs on *S. pneumoniae* phagocytosis was compared with exogenous PGE2 and Forskolin (1:100 = 41%, #, $P < 0.05$ versus AM^+Sp). Moreover, we investigated whether efferocytosis by AMs are able to inhibit the S. pneumoniae phagocytosis by scavenger receptor AI/II (SR-AI/II) and/or macrophage receptor with collagenous structure (MARCO). The inhibition of *S. pneumoniae* phagocytosis by efferocytosis was more pronounced when SR-AI/II were blocked CASR antagonist, fucoidan.
Conclusions: Conclusion: In the present study, we demonstrated that efferocytosis by AMs through of PGE2/adenylate cyclase/cAMP pathway inhibit S. pneumoniae phagocytosis and MARCO is the most important scavenger receptor involved in this suppressive effects mediated by efferocytosis.

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P0240

Efferocytosis-induced macrophage regulatory phenotype is dependent on PAFR and CD36, lipid raft integrity and COX2-dependent IL-10 production

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Purpose/Objective: We have previously shown that PAFR interacts with scavenger receptors and that phagocytosis of apoptotic cells requires both receptors. Here, we investigated whether interaction of PAFR with scavenger receptor CD36 affects macrophage phenotype.
Materials and methods: Bone marrow-derived macrophages (BMDM) from C57BL/6 mice were treated with WEB2086 (50 μM), CD36 blocking antibody (1 μg/ml), piceatannol (50 μM), β-cyclodextrin (1 mM), nimesulide (30 μM), GW9662 (20 μM) or cytochalasin D, (5 μM), 30 min before incubation with apoptotic thymocytes (annexin V^+ after 5 Gy irradiation). After 18 h, BMDM were stimulated or not with LPS (10 ng/ml) for 24 h.
Results: BMDM that ingested apoptotic cells expressed PAFR, mannose receptor, arginase-1 and IL-12 and IL-10. The PAFR antagonist WEB2086 reduced both cytokines but preferentially IL-10 (55%). Inhibition of COX-2 by nimesulide had similar effect on IL-10 production (56% of inhibition). In LPS-stimulated BMDM, although the levels of IL-10 and IL-12 were increased, WEB2086 and nimesulide reduced only IL-10 production. Furthermore, IL-10 levels were also reduced by blocking CD36, by disrupting lipid rafts with β-cyclodextrin and by inhibiting phagocytosis with cytochalasin D. Inhibitors of Syk (piceatannol) and PPARγ (GW9662) also reduced IL-10.
Conclusions: These results suggest that, for apoptotic cells to induce a regulatory phenotype ($IL\text{-}10^{high}$ and $IL\text{-}12^{low}$), it requires ingestion, engagement of PAFR and CD36 and lipid rafts integrity. The IL-10 is dependent on COX-2, Syk and PPARγ activation.
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P0241

Escherichia coli heat-labile enterotoxin affects stability of mouse embryo by inducing Th1 cytokine production and activating inflammasome

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Purpose/Objective: To analyze the effects of recombinant *Escherichia coli (E. coli)* heat-labile enterotoxin (LT) on mouse embryo stability, cytokine production and inflammasome activation.
Materials and methods: The recombinant LT was prepared with *E. coli* expression system. The pregnant mice was treated *in vivo*with LT to investigate the effects of LT on embryo stability and cytokine production. The macrophages were treated*in vitro* with LT to investigate the effects of LT on inflammasome activation.
Results: The results showed LT could significantly decreased mouse embryo survival rate. The Th1 cytokine (IFN-γ, IL-2) levels in the mouse uterus were significantly increased, the Th2 cytokines (IL-4, IL-10) were not significantly changed, which indicate that LT can shift Th1/Th2 immune balance to Th1 immune response, which is considered as a contributor to embryo instability. The results also showed that LT could enhance IL-1β production in the mouse uterus, which suggests that LT can activate inflammasome which promotes pro-IL-1β maturation and secretion from monocyte/macrophage, resulting in inflammatory reaction in the uterine tissue. To verify LT activity for inflammasome activation, we stimulated LPS-primed mouse macrophage (B6 cells) *in vitro* with LT and its different subunits (LT-A and LT-B), respectively. The results showed that both LT and LT-A subunit could enhance IL-1β secretion, and LT showed more powerful enhancement than that of LT-A subunit in IL-1β secretion, however, LT-B subunit did not enhance IL-1β secretion. These results demonstrated that LT-A subunit plays a critical role in activating inflammasome.
Conclusions: These results indicate that LT affects embryo stability is mainly related to its dysregulation on immune response of pregnant mice.

P0242

Evaluation of the potency of CD137-activated macrophages in inhibiting tumor cell growth

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Purpose/Objective: Macrophages are hematopoietic cells which play a pivotal role in immune defense. Macrophages can phagocytose pathogens and even entire cells. Also, by releasing toxic compounds including reactive oxygen intermediates or by expressing apoptosis-inducing cytokines such as tumor necrosis factor (TNF) or TRAIL, macrophages can kill bacteria and tumor cells.

Macrophages express the ligand for CD137 on their cell surface which induces their activation, via reverse signaling. In this study, we aim to investigate whether CD137 ligand signaling can enhance the ability of macrophages to interfere with the growth and /or viability of cancer cells.
Materials and methods:

1. Peripheral human monocytes were cultured for up to 10 days. Macrophage colony-stimulating factor (M-CSF) was added to differentiate the monocytes into macrophages.
2. The macrophages were treated with recombinant immobilized CD137-Fc protein overnight before being co-cultured with MCF-7, a human breast cancer cell line, or with HeLa, a human cervical cancer cell line.
3. Proliferation of cancer cells was quantified by ^{3}H-thymidine incorporation and CFSE dilution.
4. The cell cycle of the cancer cells was analysed by flow cytometry after 7-AAD staining.
5. Levels of TNF, M-CSF and interleukin-6 (IL-6) were measured by ELISA.

Results: Macrophages that had been pre-treated with immobilized CD137-Fc protein significantly reduced the proliferation of MCF-7 and HeLa cells, and caused a higher percentage of MCF-7 and HeLa cells with a reduced DNA content, which would be consistent with an increased incidence of apoptosis. Also, higher levels of TNF, M-CSF and IL-6 were present in the supernatants of these macrophage tumor cell co-cultures.
Conclusions: Our study shows that activation of macrophages by recombinant CD137 protein which induces reverse CD137 ligand signaling enables macrophages to suppress proliferation of cancer cells *in vitro*. Future studies will evaluate whether CD137 protein might be able to enhance the cytolytic activity of macrophages.

P0243

Expression pattern of sirtuins in innate immune cells and influence of Sirtuin 1/2 inhibition on innate immune responses to Toll-like receptor ligands and to infection

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Purpose/Objective: The family of histone deacetylases comprises 18 members in mammals, among which seven sirtuins (SIRT1-7). Sirtuins

are NADP-dependent enzymes that have been involved in the control of cell metabolism, proliferation and survival. The expression pattern of sirtuins and their influence on host response to microbial infection remain largely unknown. The aim of the study was to analyze the expression of SIRT1-7 and to address the effects of SIRT1/2 inhibition on innate immune responses *in vitro* and *in vivo*..

Materials and methods: *in vitro*: Bone marrow (BM), BM-derived macrophages (BMDMs) and dendritic cells (BMDCs) and RAW 264.7 and J774.1 macrophage cell lines were stimulated for 0, 2, 6 and 18 h with LPS, Pam_3CSK_4 and CpG ODN. SIRT1-7 mRNA was quantified by real time-PCR. TNF was measured by ELISA. *In vivo*: BALB/c mice were challenged with LPS (350 μg i.p.) with or without a SIRT1/2 inhibitor. Blood and organs were collected after 0, 1, 4, 8 and 24 h to quantify SIRT1-7 and TNF. Mortality was assessed daily.

Results: Bone marrow, macrophages and DCs express, in order of abundance, SIRT2 > > SIRT1, SIRT3 and SIRT6 > SIRT4, SIRT5 and SIRT7. Microbial products decrease the expression of all sirtuins except SIRT6 in a time dependent manner in BMDMs (0−24 h). SIRT2 is the most expressed sirtuin also in the liver, kidney (together with SIRT3) and spleen. Upon LPS challenge, SIRT1, SIRT3, SIRT4 and SIRT7 mRNA levels decrease in the liver (from 4 h to 24 h), whereas SIRT1-7 mRNA levels decrease within 1 h in both kidney and spleen. Pharmacological inhibition of SIRT1/2 decreases TNF production by macrophages stimulated with LPS, Pam_3CSK_4 and CpG ODN ($n = 6$; $P < 0.001$). In agreement, prophylactic treatment with a SIRT1/2 inhibitor decreases TNF production ($n = 8$; $P = 0.04$) and increases survival ($n = 13$, $P = 0.03$) of mice challenged with LPS.

Conclusions: Sirtuins are expressed in innate immune cells. Inhibition of SIRT1/2 activity decreases cytokine production by macrophages and protects from endotoxemia, suggesting that sirtuin inhibitors may represent novel adjunctive therapy for treating inflammatory disorders such as sepsis.

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P0245

Fc receptor-induced differentiation of Ly6Chi monocytes to Ly6Cint patrolling-like monocytes

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Purpose/Objective: *Purpose/objective.* Monocytes can be sub-divided into two subsets that are defined by their Ly6C and CX_3CR1 expression. $Ly6C^{hi}$ CX_3CR1^{int} are said to be the pro-inflammatory subset that are released from the bone marrow in a CCR2 dependent manor. $Ly6C^{int}$ CX_3CR1^{hi} monocytes can be seen patrolling along blood vessels and some say these cells give rise to a more anti-inflammatory macrophage or dendritic cell. Though there is much debate over the origins and function of the two monocyte subsets. Studies by our group have shown that staphylococcus protein A (SpA) interacts with IgG *in vivo* to form small immune complex (SIC) that drive anti-inflammatory pathways. Theses complexes interact in an FcgRI-dependent fashion *in vivo* with both monocytes and macrophages. They have been shown to skew macrophage to an anti-inflammatory phenotype. The profound effect of SIC on macrophage phenotype made us question how SIC interaction with monocyte subsets and alter their phenotype.

Materials and methods: C57BL/6 mice were treated with either 500 mg of SpA or ovalbumin. In adoptive transfer studies, $Ly6C^{hi}$ monocytes from the bone marrow of CX_3CR1-GFP mice were sorted and 2×10^6 cells were adoptively transferred into recipient C57BL/6 prior to treatment. Twenty-four hours later blood, bone marrow and spleen were harvested and examined by flow cytometry.

Results: Treatment with SpA showed a significant decrease in the proportion of $Ly6C^{hi}$ monocytes and a significant increase in the proportion of $Ly6C^{int}$ monocytes in the blood in relation to the control group, no changes were seen in other tissues. Suggesting an either an altered differentiation of $Ly6C^{hi}$ into $Ly6C^{int}$, or an increase mobilization of $Ly6C^{int}$. To investigate the form hypothesis, GFP-$Ly6C^{hi}$ monocytes were adoptively transfer into C57BL/6. In SpA treated mice there was a significant shift in the profile of blood $LyC6^{hi}$ GFP^+ monocytes to a $Ly6C^{int}$ phenotype. This change was also associated with an up-regulation of CX_3CR1 and a down-regulation of GR-1 and CD64. This phenotype was also seen in the spleen and bone marrow though far less GFP cells had trafficked to these sites.

Conclusions: SIC via an Fc receptor-mediated interaction induces the differentiation/maturation of blood $Ly6C^{hi}$ monocytes into $Ly6C^{int}$ monocytes. This increase in the $LyC6^{int}$ population has the potential to modify the responsiveness of the immune system it inflammatory challenges.

P0246

Gene Expression Analysis of Pig Macrophages Reveals Similarities to Humans

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Purpose/Objective: The laboratory mouse is an imperfect model in which to study human innate immunity. The larger size of the pig, and closer evolutionary distance to humans, offer several advantages. In particular, it is more straightforward to access large numbers of lung macrophages. In this study, we compared the gene expression profiles of macrophages from different breeds and compartments; alveolar (AM), bone-marrow-derived, monocyte subsets and monocyte-derived macrophages.

Materials and methods: We isolated a large number of mononuclear cells from the lung, bone-marrow and blood of 25 pigs from five breeds. Phagocytosis, TNF production and the expression of macrophage markers were characterised. We used a newly-generated and annotated pig expression array to characterise gene expression and the response to lipopolysaccharide.

Results: Isolated macrophage populations from pigs resemble those of humans. All type of macrophages expresses CD16, the LPS co-receptor CD14, CD172a. CD163 expression defined a subset of monocytes, and was expressed inversely with CD14. It was retained on alveolar macrophages (AM). Alveolar macrophages had a specific gene expression profile that included high levels of many C type lectins. Like peripheral blood monocytes, AM comprised two two subpopulations that differed in adherence, LPS response, phagocytosis and expression of CD163. $CD14^{++}$ monocytes resembled $CD14^{++}$ human monocytes in the expression profile. Human and pig macrophages also shared expression of LPS-inducible genes (STAT4, IDO, CCL20, Cyp27B1) that are not induced in mouse macrophages, and failed to induce iNOS. Pig breeds showed no great differences in response to LPS. The few genes differentially expressed included cytochrome CYP3A29, the metalloproteinase MMP1, STEAP4 and the N-Myc interactor NMI.

Conclusions: We have isolated and characterised the gene expression profiles of pig macrophages in multiple differentiation and activation states. The data support the use of the pig as a model of innate immunity that more closely resembles humans, and is economically important in its own right.

P0247

High-resolution transcriptome of human macrophages

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Purpose/Objective: Macrophages are dynamic cells integrating signals from their microenvironment to develop specific functional responses. Microarray-based transcriptional profiling has established transcriptional reprogramming as an important mechanism for signal integration and cell function of macrophages yet current knowledge on transcriptional regulation is far from complete. RNA sequencing (RNA-seq) is ideally suited to fill this need but also to discover novel marker genes, an area of great need particularly in human macrophage biology.

Materials and methods: Applying RNA-seq, we provide a high-resolution transcriptome profile of human macrophages under classical (M1-like) and alternative (M2-like) polarization conditions and demonstrate a dynamic range exceeding observations obtained by previous technologies, resulting in a more comprehensive understanding of the transcriptome of human macrophages.

Results: In addition, we were able to detect differential promoter usage, alternative transcription start sites, and different coding sequences for 57 gene loci in human macrophages. Moreover, this approach led to the identification of novel M1-associated (CD120b, TLR2, SLAMF7) as well as M2-associated (CD1a, CD1b, CD93, CD226) cell surface markers.

Conclusions: Taken together, these data support that high-resolution transcriptome profiling of human macrophages by RNA-seq leads to a better understanding of macrophage function and will form the basis for a better characterization of macrophages in human health and disease.

P0248

Human CD1a$^+$ dendritic cells mediate potent anti-viral immune responses via RIG-I and MDA5 signalling

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Purpose/Objective: Cytosolic RIG-I-like helicases (RLH) are pattern recognition receptors involved in type I interferon production and antiviral immunity. The heterogeneous population of dendritic cells (DCs) act as professional antigen presenting cells in both lymphoid and non-lymphoid tissues to coordinate innate and adaptive immunity. This study focuses to the comparative analysis of the expression, functional activities and signalling cascades associated to RLH in *in vitro* generated and previously characterized CD1a$^+$ and CD1a$^-$ human monocyte-derived dendritic cell (moDC) subsets.

Materials and methods: Human moDCs were differentiated from blood-derived monocytes. CD1a$^-$ and CD1a$^+$ DCs were sorted by FACS DiVa. Relative mRNA expressions were analyzed by real-time Q-PCR. Protein expressions were measured by Western blot, the levels of secreted cytokines were detected by ELISA. Small interfering RNA (siRNA) was introduced by electroporation.

Results: Our results revealed that the expression of RLH genes and proteins as well as the activity of the coupled signalling pathways were significantly higher in the CD1a$^+$ subset than in its developmentally related but phenotypically and functionally distinct CD1a$^-$ counterpart. Specific activation of RLH in moDCs by polyI:C or influenza virus induced the secretion of IFNβ via IRF3, and pro-inflammatory cytokine responses through TLR3. Ligand-induced RLH-mediated signalling in CD1a$^+$ moDCs was shown to be indispensable for both priming naïve CD8$^+$ T lymphocytes and inducing potent influenza virus-specific cellular immune responses as demonstrated by RIG-I/MDA5 silencing that abrogated these functions. We also provide evidence that this migratory moDC subset can be detected in human tonsil and reactive lymph nodes.

Conclusions: We demonstrated the DC subset-specific activation of RLH and identified CD1a$^+$ moDCs as potent antigen presenting and highly inflammatory cells due to their specialized functional activities Targeting the differentiation and RLH ligand-specific activation of the CD1a$^+$ DC subpopulation may offer new tools for improving the efficacy of anti-viral vaccines.

P0249

Human monocytes express functional trail receptors and are susceptible to trail-induced apoptosis

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Purpose/Objective: In several tumor types, the presence of Tumor-Associated Macrophages (TAM) correlates with poor clinical outcome and resistance to conventional treatment.TAM promote disease progression by supporting cancer cell survival, proliferation and invasion. Furthermore, TAM and related myeloid cells (Tie2$^+$ monocytes and MDSC) trigger neo-angiogenesis and suppress adaptive immune responses. Targeting of tumor macrophages is considered a promising therapeutic strategy to be combined with other conventional anti-tumor treatments. It is known that in tumor cells the activation of the apoptotic pathway via death receptors represents a good therapeutic strategy. For this purpose we decided to study the expression of death receptors and ligands and their functional activity in leukocyte population.

Materials and methods: We checked expression of death receptors in whole human blood by FACS analysis. We treated human monocytes with a recombinant trail protein and we checked its functionality by Annexin V$^+$ apoptosis and caspase 8 expression by citofluorimeter analysis.

Results: We show in this study that blood monocytes express the death receptors for theTumor necrosis factor-Related Apoptosis Inducing Ligand (TRAIL), a cytokine known to induce apoptosis in a wide variety of transformed cell lines but not normal cells. R1 (TNFRSF10A) and R2 (TNFRSF10B) are significantly expressed by monocytes while the non-signaling decoy receptor R3 (TNFRSF10C) is virtually absent. In marked contrast, lymphocytes and neutrophils express high levels of R3 and not of R1 and R2, implying that monocytes are selectively susceptible to this killing mechanism. Recombinant TRAIL protein induced death of human monocytes *in vitro* via caspase 8 activation and Annexin V$^+$ apoptosis, which was specificallyinhibitedby blocking anti-TRAIL receptor antibodies.

Conclusions: Along the current concept that modulation of the host micro-environment, of macrophages in particular, may result in enhanced response to anti-cancer therapies, we propose thatmonocytes/macrophages can be targeted by TRAIL.

P0250

Human scavenger protein Spalpha regulates key macrophage lipid homeostasis events that lead to atherosclerosis development

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Purpose/Objective: Macrophage Secreted Protein α (Spα/CD5L) belongs to the Scavenger Receptor Cysteine Rich Superfamily (SRCR-SF). In the mouse, its expression is under control of the Nuclear Receptor (NR) transcription factors LXR and RXR. Murine Spα is considered a pro-atherogenic factor by protecting macrophages from the apoptotic effects of oxidized lipids. To date, the sole information available on its human counterpart (hSpα) is that it is detected in human atherosclerosis lesions. The objective of this study was to assess the contribution of hSpα to the macrophage events that lead to atherosclerosis.
Materials and methods: THP1 cells stably expressing hSpα and human macrophages differentiated from healthy donor peripheral blood monocytes treated with recombinant hSpα have been studied.
Results: Our results show that, as in mice, hSpα plays an antiapoptotic effect to modified LDL (mLDL) in macrophages. Furthermore, hSpα increased foam cell formation as assessed by Oil-Red-O staining and total and esterified cholesterol content quantification by thin layer chromatography. Flow cytometry studies showed that hSpα increases fluorescently labelled mLDL uptake by macrophages. This could be explained by an increase on the mRNA levels of the canonical mLDL uptake receptors CD36 and SRA. Also, hSpα did not affect cholesterol efflux during 3H-labelled cholesterol reverse transport to plasma. Real time PCR and western blot analyses revealed no differences between human and mice in terms of regulation by OxLDL and LXR/RXR synthetic agonists. Yet, hSpα protein synthesis and secretion are tightly regulated.
Conclusions: Taken together our data suggest that hSpα retains the antiapoptotic role and that its expression is under control of the NR. We further demonstrate that hSpα contributes to additional key aspects of the role of macrophages in lipid homeostasis, such as foam cell formation due to an imbalance between cholesterol uptake and efflux. Our findings provide valuable information for its potential therapeutic intervention for the treatment of atherosclerosis.

P0251

IFN-a inhibits the type I immune response of human monocytes

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Purpose/Objective: Both IFN-α and IFN-γ display immunomodulatory effects. IFN-γ is the main mediator of Th1 responses essential in the control of infections by intracellular bacteria, such as non-tuberculous Mycobacteria, while IFN-α plays a role in viral infections. Virulent Mycobacterium tuberculosis strains may induce IFN-α and thereby interfere with an effective Th1 response against this bacterium. Herein we investigate how IFN-α modulates type I immune responses by monocytes.
Materials and methods: Human blood $CD14^+$ monocytes were isolated and stimulated overnight with various concentrations of IFN-α and IFN-γ in the presence or absence of LPS. The IFN-γ induced expression of CD54 and CD64 was measured by FACS and the production of type I cytokines by Elisa. Next, we investigated the mechanism by which IFN-α inhibits the type I immune responses. We examined the influence of IFN-α on the expression of the IFN-γ receptor and on the IFN-γ-induced signal transduction, by FACS and Western blot. To investigate a role for protein arginine methyltransferase-1 (PRMT-1) in the inhibition of IFN-α we used a competitive PRMT inhibitor.
Results: IFN-α reduced the IFN-γ-enhanced CD54 and CD64 expression up to threefold. The LPS-induced IL-12p40 production was fivefold reduced by IFN-α. The IL-12p40, TNF and IL-1β production induced by the combination of IFN-γ and LPS were seven-, four- and threefold reduced by IFN-α, respectively.

To study the effects of IFN-α on IFN-γ signalling we determined the kinetics of STAT1 and STAT2 phosphorylation and found that IFN-α interferes directly with the IFN-γ-induced STAT1 phosphorylation. The cell surface expression of IFN-γR1 was gradually reduced after 2 h of stimulation with IFN-α to <25% it's starting value. In addition, we revealed a role for PRMT1, which is associated with the IFN-α receptor, in the inhibitory effects of IFN-α.
Conclusions: In conclusion, IFN-α strongly inhibits type I immune response of monocytes via diverse modes of action.

P0252

IL-15-dependent $NKp46^+$ innate lymphoid cells control intestinal inflammation via CCL3-CCR1-dependent recruitment of inflammatory monocytes

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Purpose/Objective: Interleukin 15 (IL-15) is a pleiotropic cytokine with a large range of functions at the interface between innate and adaptive immunity. Its contribution during intestinal inflammation was investigated by using a model of acute ileitis induced by oral administration of *Toxoplasma gondii* (*T. gondii*) in C57BL/6 wild type (WT) mice.
Materials and methods: The role of IL-15 was assessed by studying the severity of ileitis WT and $IL\text{-}15^{-/-}$ mice.
Results: Ileitis was markedly attenuated in $IL\text{-}15^{-/-}$ mice but the parasite load was not modified, indicating that IL-15 enhanced the severity of intestinal inflammation induced by *T. gondii*. The pro-inflammatory effect of IL-15 did not depend on IEL activation. In contrast, IL-15 promoted the recruitment of $CCR1^+$ inflammatory monocytes (IM) into the inflamed gut. IMs produced high levels of inflammatory cytokines which lead to epithelial damage. $CCR1^+$ IMs were attracted by CCL3, produced by a subset of intestinal $NKp46^+$ innate lymphoid cells. A non-hematopoietic source of IL-15 controlled the development and function of these intestinal $NKp46^+$ cells in response to inflammatory cytokines, and notably to IL-18 during ileitis induced by *T. gondii* infection.
Conclusions: Together these data delineate a novel function for a IL-15-dependent subset of intestinal $NKp46^+$ cells as an important source of CCL3, promoting the recruitment of $CCR1^+$ inflammatory monocytes. Preliminary evidence suggests that this pathway may also operate in Crohn disease.

P0253

Immobilized immune complexes recruit CD16-positive human blood dendritic cells

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Purpose/Objective: The deposition of immune complexes (ICs) in the skin is a critical pathogenic event in the promotion of autoimmune inflammatory responses. Fc receptor-mediated interaction of these ICs with the machinery of dendritic cells (DCs) contributes to IC-triggered inflammation and tissue damage. Among human blood DCs we previously identified the CD1c- $CD11c^+$ slanDCs, identifiable by expression of the carbohydrate modification 6-sulfo LacNAc. SlanDCs stand out by their high level expression of the low affinity Fc gamma receptor III (CD16) and exert powerful proinflammatory effects by serving as a major and early source of IL-12 and TNF-alpha.
Materials and methods: We produced small soluble and fluorescent ICs and flow cytometrically measured the binding of these ICs to human blood DCs and monocytes. Furthermore, we applied a flow chamber adhesion assay and time-lapse video microscopy to measure the arrest function of blood leukocytes to immobilized immune complexes under conditions of physiological shear stress.
Results: We show that marked expression of CD16 equips slanDCs with an outstanding capacity to capture small soluble ICs. We then demonstrate that ICs alone are highly efficient in mediating the firm arrest of slanDCs under conditions of physiological shear stress. In stark contrast, other human blood DCs (plasmacytoid DCs and $CD1c^+$ DCs) or T cells completely failed to adhere immobilized ICs. By selectively blocking the FcγRs on slanDCs, we clearly show that their shear-resistant adherence to immobilized ICs critically depends on CD16, while CD32 (FcγRII) expression is irrelevant. The existence of this CD16/IC-mediated DC recruitment at the endothelial interface is furthermore supported by data showing enhanced arrest of slanDCs on monolayers of human dermal microvascular endothelial cells that were pre-incubated with anti-endothelial cell IgG antibodies.
Conclusions: Taken together, we have shown that CD16 expressed by slanDCs mediates 1) highly efficient capture of soluble ICs and 2) efficient shear-stress-resistant adhesion of circulating slanDCs to immobilized ICs. These data provide evidence for a novel conduit of rapid FcR-dependent recruitment of DCs in IC-mediated tissue inflammation.

P0254

Immune response to diphtheria toxin-mediated depletion complicates the use of the CD11c-DTRtg model for studies of bacterial gastrointestinal infections

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Purpose/Objective: Dendritic cells (DCs) play an important role in the immune defense against pathogens, as they alert the immune system to invaders, and are responsible for the activation and control of both innate and adaptive immune responses. In this study, we aimed to investigate the role of DCs during early *Y. pseudotuberculosis* infection by the use of a mouse DC-depletion model.
Materials and methods: The CD11c-DTR^{tg} model has been extensively used to study the role of DCs during steady-state and infection conditions. The CD11c-DTR^{tg} mice express a primate DTx receptor (DTR) under the control of the CD11c promoter (Itgax), which allows for conditional knock-out of $CD11c^{high}$ cells, including most DC populations, by injection of DTx. *Yersinia* infection was monitored by IVIS and the immune response in the mice was evaluated by immunohistochemistry, flow cytometry and a multiplex cytokine assay.
Results: Infecting DC-depleted mice orally with *Y. pseudotuberculosis* results in a markedly reduced level of infection compared with infection of non-depleted mice, suggesting an important role for DCs in establishment of the infection. However, investigations of the immune status in uninfected mice after DTx-treatment revealed that this lower bacterial colonization is due to a DTx-mediated immune response leading to recruitment of PMNs in Peyer's patches and increased levels of keratinocyte-derived cytokine in blood.
Conclusions: DTx-mediated depletion leads to an immune response that efficiently reduces the initial colonization of *Y. pseudotuberculosis* in Peyer«s patches. It is possible that the recruited PMNs eliminate the majority of the infecting bacteria or that the induced inflammatory response in the Peyer's patches causes restriction of bacterial entry.

P0255

Impact of Sirtuin 2 knockout on innate immune responses

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Purpose/Objective: Histone deacetylases (HDACs) deacetylate histones and transcriptional regulators thereby affecting numerous biological functions. Seven mammalian sirtuins (SIRT1-7) constitute the NAD-dependent class III subfamily of HDACs. Sirtuins are the center of great interest due to their regulatory role in the control of metabolism, ageing and age-related diseases. Up to now, little is known about the influence of sirtuins on immune responses, and nothing about the role of SIRT2. The aim of the study was to analyze the influence of SIRT2 knockout on immune cell development and innate immune responses *in vitro* and *in vivo*.
Materials and methods: SIRT2 germline knockout were produced on a C57BL/6J background. The cellularity of thymus and spleen was assessed by flow cytometry ($n = 3$). Bone marrow derived macrophages (BMDMs) and dendritic cells (BMDCs) and splenocytes were stimulated with LPS, Pam_3CSK_4 lipopeptide, CpG ODN, *E. coli*, *S. aureus*, TSST-1, SEB, anti-$CD3^+$ CD28 and concanavalin A ($n = 3–8$). TNF, IL-2, IL-6, IL-12p40 and IFNγ production, SIRT1–7 and CD40 expression, and proliferation were quantified by real time-PCR, ELISA, flow cytometry and H^3-thymidine incorporation. Mice ($n = 6–16$) were challenged with LPS, TNF/D-galactosamine, *E. coli* and *K. pneumonia* titrated to cause either mild or severe infections or shock. Blood was collected to quantify cytokines and bacteria. Mortality was checked regularly.
Results: SIRT2 is the most expressed sirtuin in macrophages and myeloid DCs. To test whether SIRT2 impacts on innate immune responses, we generated SIRT2 germline knockout mice. $SIRT2^{-/-}$ mice born at the expected Mendelian ratio and develop normally. The proportions and absolute numbers of DN1-4, DP and SP thymocytes, and of T-cells (DN and SP, naïve and memory), B-cells (immature and mature), DCs (cDCs and pDCs) and granulocytes in the spleen are similar in $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice. $SIRT2^{+/+}$ and $SIRT2^{-/-}$ BMDMs, BMDCs and splenocytes produce cytokines (RNA and protein), upregulate CD40, and proliferate to the same extent. $SIRT2^{+/+}$ and $SIRT2^{-/-}$ mice respond similarly (cytokine blood levels, bacterial counts and mortality) to non-severe and lethal endotoxemia, *E. coli* peritonitis, *K. pneumonia* pneumonia and TNF-induced shock.
Conclusions: SIRT2 knockout has no dramatic impact on the development of immune cells and on innate immune responses *in vitro* and *in vivo*. Considering that SIRT2 may participate to control metabolic homeostasis, we are currently assessing the impact of SIRT2 deficiency on innate immune responses under metabolic stress.

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P0256

in vitro differentiation of negatively isolated primary monocyte: an adequate model system to study infections of human macrophages by Listeria monocytogenes

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Purpose/Objective: Macrophages are an important defence mechanism against intracellular pathogens such as *Listeria monocytogenes* (*Lm*). Depending on their surface marker expression and immunological function, macrophages have pro-inflammatory or anti-inflammatory characteristics. Most studies to date on *Lm* infections of macrophages have been conducted in cell lines or murine bone-marrow-derived macrophages. Several differences have been reported between murine and human macrophages. In the present study we thus evaluate different methods to generate macrophages from primary human monocytes to investigated *Lm* infections.
Materials and methods: *In vitro* these two subpopulations can be derived from $CD14^+$ monocytes isolated from human blood by cultivation in the presence of either granulocyte macrophage colony-stimulating factor (GM-CSF) or macrophage colony-stimulating factor (M-CSF) to generate inflammatory GM-CSF monocyte derived macrophages (GM-MDM) and anti-inflammatory M-CSF monocyte derived macrophages (M-MDM). We used both negative as well as positive selection to isolate $CD14^+$ monocytes from human blood samples. For comparison PMA-derived THP-1 macrophages were used. Cells were subsequently incubated with either GM-CSF or M-CSF and analyzed for surface expression of the macrophage differentiation markers CD163 and CD206.
Results: While PMA-stimulated THP-1 macrophages were negative for these two markers, GM-MDM stained high for CD206 and M-MDM were positive for CD163 following both positive and negative isolation methods. However, when analyzed for phagocytosis of *Lm,* a striking difference was observed between macrophages derived from monocytes isolated by the two methods. After positive selection both GM-MDM and M-MDM were highly phagocytic towards the pathogen. By contrast, when isolated by negative selection only M-MDM displayed high phagocytosis. Moreover, high non-specific phagocytosis of latex beads by M-MDM was observed irrespective of the method used for isolation of monocytes. High phagocytic activity in GM-MDM from negatively selected monocytes could be induced by addition of the CD14-antibody (short term and 7 days) used for positive selection.
Conclusions: Collectively the results suggest that positive selection using a CD14-antibody alters the phagocytic behavior of GM-MDM towards *Lm*. Human macrophages derived from primary monocytes by negative selection are proposed as the most suitable model to study *Lm* infection of macrophages.

P0257

Increased inflammation modifies RA monocyte susceptibility to TRAIL-induced apoptosis

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Purpose/Objective: The autoimmune disease rheumatoid arthritis (RA) is characterized by the production of proinflammatory monocytic cytokines like TNF alpha and IL-1 beta. Neutralization of TNF alpha with anti-TNF antibodies is a critical therapeutic approach, which has a strong influence on disease activity in RA patients. In a previous study we already have shown that monocytes from patients with rheumatoid arthritis are less sensitve and sometimes resistant against TRAIL-induced apoptosis. In the following study, the influence of disease activity of RA patients on TRAIL-induced apoptosis of monocytes should be analyzed. Furthermore the importance of an anti-TNF therapy in this context will be studied.
Materials and methods: Monocytes of 24 RA patients were assessed for TRAIL induced apoptosis and cytokine production before (BL) and after 4, 12 and 24 weeks of anti-TNF treatment. Therefore monocytes were isolated from the peripheral blood of RA patients were incubated with 100 ng/ml TRAIL for 16 h. Subsequently TRAIL induced apoptosis was measured via annexinV/PI staining. Production of IL-8 was analyzed by ELISA. Expression levels of all TRAIL receptors and mTRAIL were detected by flow cytometry. Evaluation of disease activity by using DAS 28, ESR and CRP takes place at every time point of the treatment periode.
Results: The analysis of TRAIL induced apoptosis of RA monocytes before anti-TNF therapy show that monocytes from patients with a high disease activity undergo TRAIL induced apoptosis while monocytes from patients with low disease activity are did not. During the study a decreasing disease activity in patients under threapy was observed. Simultaneously the TRAIL induced apoptosis decreases in positve correlation with the disease activity. Furthermore, it was shown that RA monocytesproduce increased amounts of IL-8 production after TRAIL stimulation. This production was significantly inhibited during anti-TNF therapy. The disease activity of the monocyte donors has no effect on the spontaneous IL-8 production andon the TRAIL induced IL-8 production. Of all the donors monocyte cell surface expression of all TRAIL receptors and of mTRAIL was determined to BL and at different time points during therapy. It was shown that anti-TNF treatment leads to inhibition of TRAIL R1 and R3 expression.
Conclusions: anti-TNF therapy inhibits inflammation induced amplification of proinflammatory effects of TRAIL in RA monocytes.

P0258

Induction of peroxisome proliferator-activated receptor (PPAR) -g in macrophages by human amniotic membrane

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Purpose/Objective: To study the observed immune suppressive functions of amniotic membrane (AM) on macrophages.
Materials and methods: Apoptosis of macrophages was studied by flow cytometric analysis by using the annexin V/7-AAD system. Cytokines were measured by ELISA and the expression of nuclear factor (NF)-κB and PPAR-γ by western blot. The activity of arginase was determined by bioassay. The expression of costimulatory molecules and PPAR-κ was measured by flow cytometric observation. Accumulation of lipids in the cytoplasma was observed by Oil red O staining or Sudan black B.
Results: After coculture with AM macrophages show a decreased NF-κB expression, cytokine secretion (TNF-α, IL-6, IL-10, IL-12), expression of costimulatory molecules (CD80, CD86, CD40), phagocytosis, proliferation, and viability. The expression of CD206, CD204, CD163, and CD68, lipid accumulation in the cytoplasma, PPAR-γ expression and arginase activity was increased.
Conclusions: We conclude that the action mechanisms of AM is associated with an activation of the PPAR-γ pathway and engagement of the lipid metabolism in macrophages, resulting in a modulation of classically activated macrophages into alternative activated macrophages or macrophage cell death upon activation.

P0259

Inhibition of inflammasome activation in human macrophages by highly pathogenic avian influenza viruses caused by missing M2 protein expression

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Purpose/Objective: Purpose/Objective: Infections of the human host by highly pathogenic avian influenza viruses (HPAIV) are characterized by development of a cytokine storm and systemic spreading of infection. Macrophages as part of the first barrier of defense against systemic infections are important producers of cytokines and therefore most likely to be involved in the emergence of a cytokine storm.
Materials and methods: Materials and methods: We analyzed human blood-derived macrophages infected with low pathogenic human A/PR8/34 (H1N1) and HPAIVs A/FPV/Bratislava/79 (H7N7) and A/Thailand/1 (KAN-1)/2004 (H5N1) in a genome-wide microarray study and confirmed results in quantitative Real-time PCR experiments. Immunofluorescent staining and Western Blot were used for analysis of viral protein expression.
Results: Results: We found a surprisingly low inflammatory and antiviral response of macrophages infected with HPAIV in contrast to those infected with human H1N1. Immunofluorescence and Western Blot analyses revealed that virus replication and expression of viral proteins in the host cell take place with one exception. Viral protein M2 is not expressed in macrophages in case of HPAIV infection. As M2 serves as second signal for inflammasome activation in macrophages, we found a significant reduction of IL-1β protein expression.
Conclusions: Conclusions: Missing M2 expression therefore leads to subsequent suppression of an important part of the inflammatory and antiviral response and thus enables HPAIV to bypass effective immune responses of one of the most important cell types of the innate immune system. Consequently, systemic spreading of HPAIV infection is facilitated.

P0260

Intestinal CD8a$^+$ dendritic cells constitutively migrate from the intestinal lamina propria to the mesenteric lymph node

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Purpose/Objective: Dendritic cells (DCs) are central to the initiation of all adaptive intestinal immune responses. They transport antigen from the periphery to the mesenteric lymph nodes (MLNs) where they prime naïve T cells. Previous work has focused on a CD103$^+$ CD11b$^+$ tolerogenic subset of DCs migrating from the intestinal Lamina Propria (LP) to the MLN; however, we have recently identified four distinct subsets of migratory DCs including a subset of CD103$^+$ CD8α^+. In order to deepen our understanding of these subsets of migratory intestinal DCs, we sought to determine their anatomical origins. Specifically, to determine if these DCs originate from the LP or from organised lymphoid tissues in the intestine.
Materials and methods: We used two mouse models which lack specific secondary lymphoid tissues in the intestine. RORγt$^{-/-}$ mice lack Peyer's patches (PPs) and isolated lymphoid follicles (ILFs), while progeny of mice treated with Lymphotoxin-β Receptor Fusion Protein (LT-β-R-Ig) lack only PPs. In order to examine migratory DCs, we developed a technique for thoracic duct cannulation (TDC) preceded by mesenteric lymphadenectomy (MLNx), allowing the isolation of all intestinal lymph-borne DCs (LDCs). By collecting migrating lymph DCs from mice lacking specific intestinal lymphoid tissues, the anatomical origins of these DCs can be elucidated.
Results: The absence of PPs and ILFs in RORγt$^{-/-}$ mice was confirmed by immunohistochemistry on Swiss rolls made from sections of small intestine. Imaging was also performed on live intestinal tissue of LT-β-R-Ig progeny, confirming that PPs were missing in these animals. All four intestinal lymph DC subsets were present in LT-β-R-Ig progeny in comparable proportions to WT mice. However, intestinal lymph and LP from RORγt$^{-/-}$ animals contained similar proportions of only three of the four DC subsets found in WT mice; the CD103$^-$CD11b$^-$ DC subset is missing from RORγt$^{-/-}$ mice.
Conclusions: The techniques of MLNx followed by TDC allow the isolation of bona fide migratory DCs. Three subsets of LDCs, including a CD103$^+$ CD8α^+ DC subset; migrate into intestinal lymph from the LP, while CD103$^-$CD11b$^-$ DCs appear to originate from ILFs in the small intestine. Determining the anatomical origins of LDCs provides exciting new avenues for the treatment of inflammatory bowel disease and the development of oral vaccines by enabling subsets of DCs to be targeted in the intestine.

P0261

Investigating human monocyte subsets in periodontitis – potential targets for immunomodulatory treatment

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Purpose/Objective: In Periodontitis (PD), patients suffer from chronic inflammation that causes destruction of tooth supportive tissues. Evidence suggests that the persistent inflammation in PD occurs due to abnormal host innate immune responses to oral micro flora. We hypothesize that defective functional properties of the monocytes, contributes significantly to unrestrained bacteria-induced responses in PD. Monocytes are divided into three subsets, the CD14$^+$ CD16-, CD14$^+$ CD16$^+$ and CD14lowCD16$^+$ subset, with different pro- and anti-inflammatory functions. We aim to address if the composition of the subsets and their ability to migrate into tissue based on chemokine receptor expression, is altered in PD patients. In parallel we aim to develop a human 3D model of the oral mucosa with implanted monocytes, to further study their functionality at the site of inflammation.
Materials and methods: Peripheral blood was obtained from patients with PD and control individuals. Blood cell numbers and C-reactive protein (CRP) levels were analyzed. Percentages of the monocyte subsets as well as chemokine receptor expression were analyzed with multicolor flow cytometry and RT-qPCR. For the model, primary oral fibroblasts were cultured in a collagen based matrix with monocytes and the oral keratinocytes cell line OKF6-TERT2 seeded on top.
Results: The number of monocytes is significantly increased in PD patients compared to controls. In addition, PD is associated with an altered monocyte subset composition, including a decrease of the CD14lowCD16$^+$ subset. We also observed an increase in the CRP levels in PD. Finally, PD is associated with an increase of the chemokine receptor 7 (CCR7), on all monocyte subsets.
Conclusions: PD is associated with increased number of monocytes and changes in the percentages of the monocyte subsets with an altered chemokine receptor expression, potentially changing their migratory behaviour and contributing to disease progression.

P0262

Lack of PPARgamma in myeloid cells confers resistance to listeria monocytogenes infection

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Purpose/Objective: The peroxisomal proliferator-activated receptor gamma (PPARg) is a nuclear receptor that controls inflammation and immunity. Innate immune defense against bacterial infection appears to be compromised by PPARg. The relevance of PPARg in myeloid cells, that organize anti-bacterial immunity, for the outcome of immune responses against intracellular bacteria such as *Listeria monocytogenes in vivo* is unknown. In this work we address the question whether PPARg with its potent regulatory activity on innate immune functions in myeloid cells plays a regulatory role during infection with *L. monocytogenes* and whether PPARg restricts innate immunity in myeloid cells against *Listeria* infection.

Materials and methods: To investigate whether PPARg in myeloid cells influences innate immunity against bacterial infection we have generated a transgenic mice with myeloid-cell specific ablation of PPARg (LysMCre × $PPARg^{flox/flox}$) andused *Listeria monocytogenes* as a model for intracellular baceterial infection.

Results: Our data have shown that loss of PPARg in myeloid cells results in enhanced innate immune defense against *Listeria monocytogenes* infection both, *in vitro* and *in vivo*. This increased resistance against infection was characterized by augmented levels of bactericidal factors and inflammatory cytokines: ROS, NO, IFNgγ TNFα IL-6 and IL-12. Moreover, myeloid cell-specific loss of PPARg enhanced chemokine and adhesion molecule expression leading to improved recruitment of inflammatory $Ly6C^{hi}$ monocytes to sites of infection. Importantly, increased resistance against *Listeria* infection in the absence of PPARg was not accompanied by enhanced immunopathology.

Conclusions: Our results elucidate a yet unknown regulatory network in myeloid cells that is governed by PPARg and restrains both listeriocidal activity and recruitment of inflammatory monocytes during *Listeria* infection, which may contribute to bacterial immune escape. Pharmacological interference with PPARg activity in myeloid cells might represent a novel strategy to overcome intracellular bacterial infection.

P0263

Langerhans' cell migration and lymph node dendritic cell accumulation in response to chemical allergens: roles of tumour necrosis factor-a

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Purpose/Objective: Langerhans' cells (LC), the dendritic cells (DC) of the epidermis, once activated, migrate to the draining lymph nodes, where they orchestrate and regulate immune responses. We have shown previously that tumour necrosis factor (TNF)-α is required for DC accumulation in the skin draining lymph nodes after topical exposure of BALB/c strain mice to the chemical contact allergen oxazolone. TNF-α induces changes in LC, including the down-regulation of E-cadherin, which allows dissociation from neighbouring keratinocytes in the epidermis, in addition to up-regulation of matrix metalloproteinases. There are two receptors for TNF-α; TNF-R1 and TNF-R2, of which LC express only the latter. Another group has demonstrated that TNF-R2 (but not TNF-R1), is required for the accumulation of fluorescein isothiocyanate (FITC) bearing cells in the draining lymph node after topical application of the chemical allergen FITC. The aim of these experiments was to characterise the requirement for TNF-α for LC migration and DC accumulation in response to the chemical allergens dinitrochlorobenzene (DNCB) and FITC.

Materials and methods: Wild type (C57/BL6), and $TNF\text{-}R2^{-/-}$ strain mice received topical application of either DNCB or FITC, to the dorsum of both ears. After 4 or 24 h treatment with DNCB, the epidermis was isolated and stained for LC using FITC-conjugated MHC Class II antibody and analysed for LC frequency by fluorescence microscopy. Twenty-four hours after application of DNCB or FITC, auricular lymph nodes were excised and DC frequency assessed by CD11c expression, analysed by flow cytometry.

Results: 4 h after topical application of DNCB, LC migration was induced from the epidermis in both wild type and $TNF\text{-}R2^{-/-}$ strain mice. Furthermore, 24 h application of DNCB not only induced loss of LC from the epidermis, but also caused a marked increase in the number of DC in the skin draining lymph node of $TNF\text{-}R2^{-/-}$ strain mice. In contrast, FITC increased the frequency of DC in the draining lymph node of wild type, but not $TNF\text{-}R2^{-/-}$ strain mice.

Conclusions: These data suggest that, unlike FITC, DNCB-induced migration of LC from the epidermis and subsequent arrival of DC in the lymph node is not dependent on signalling through TNF-R2.

P0264

Langerhans' cells have a type 2 phenotype with regards to their Toll-like receptor ligand response and chemokine expression pattern

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Purpose/Objective: Langerhans' cells (LC) are the dendritic cell (DC) population of the epidermis and express toll like receptors (TLR) that are capable of recognising pathogen-associated molecular patterns. Upon activation, LC migrate to the lymph nodes where they initiate an immune response. Both DC and LC can secrete chemokines, of which some are associated with a type 1 or a type 2 immune response.

Materials and methods: Single cell suspensions were created from the epidermis of BALB/c strain mice. Unfractionated, LC-enriched (94% pure) and LC-depleted (predominantly keratinocyte) fractions were analysed for chemokine expression (type 1- [CXCL9 and CXCL10] and type 2- [CCL17 and CCL22] associated) by RT-PCR. Chemokine secretion by XS106 cells (an LC-like cell line derived from murine epidermis) was analysed by ELISA. TLR ligands were used to stimulate XS106 cells and surface expression of membrane markers (MHC class II, CD80, CD86 and CD40) and chemokine production was analysed by flow cytometry and ELISA, respectively. Migration of LC following intradermal injection of BALB/c strain mice with various TLR ligands was assessed in epidermal sheets by immunofluorescence staining for MHC class II.

Results: The LC-enriched and LC-depleted fractions mainly expressed type 2-associated CCL22 and type 1-associated CXCL10, respectively. XS106 cells expressed constitutively higher levels of type 2 chemokines compared with type 1 chemokines. Furthermore, XS106 cells responded differently to TLR simulation with respect to chemokine production, which was considerably lower after stimulation with type 1 TLR ligands (CpG and R-848), compared with type 2 TLR ligands (peptidoglycan [PGN] and flagellin), and was still biased towards type 2 chemokines CCL17 and CCL22. Additionally, XS106 cells up-regulated membrane marker expression after treatment with PGN and flagellin, but not CpG or R-848. However, all of the TLR ligands investigated stimulated the *in vivo* migration of LC.

Conclusions: These data suggest that keratinocytes and LC in the epidermis have preferential roles in promoting type 1 and type 2

immune responses, respectively. Therefore, the migration of LC *in vivo* after encounter with CpG or R-848 may be secondary to interaction of the TLR ligands with keratinocytes.

P0265

Linkage-specific loss of sialic acid from the aged erythrocyte as a signal for tolerogenic immune clearance

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Purpose/Objective: The typical lifespan of the human erythrocyte is 120 days. Erythrocytes are produced, and when aged, cleared at a very high rate of 2.4 million cells per second.Despite this rapid rate of uptake mediated by macrophages, the process of removal of aged erythrocytes is immunologically silent.We set out to investigate the link between how age-related changes to surface markers on the erythrocyte contribute to efficient recognition and tolerogenic clearance by phagocytes.

Materials and methods: Since erythrocytes increase in density as they age, we used a Percoll based gradients to isolate fractions of circulating erythrocytes from healthy human donors.The most dense fraction contained0.5% of all erythrocytes, which we estimate corresponds to ages between 119~120 days.

Results: Employing binding assays using a panel of lectins, including MAL-II and SNA, we show that α2, 3-linked sialic acids are well maintained during the aging process of erythrocytes while α2, 6-linked sialic acids are selectively lost in the last days of life.In contrast to natural aging, we saw complete loss of all sialic acids when aged erythrocytes were damaged, for example by osmotic shock, suggesting that this might provide a different, more immunogenic signal to phagocytes.

Conclusions: This linkage selective loss of sialic acids implies erythrocytes are subjected to specific enzymatic activity at the end of the aging process and this could contribute to their recognition by macrophages and dendritic cells, as well as the tolerogenicity of the uptake process.Loss of α2, 6-linked sialic acids would be predicted to reveal the underlying galactose sugar residue on surface glycans.This exposure can trigger phagocytosis through galactose binding receptors onmacrophages and dendritic cells, such as Galectin-1, Galectin-3 and MGL. Conversely, selective maintenance of α2, 3-linked sialic acids on erythrocytes can potentially engage inhibitory receptors on phagocytes such as members of the siglec family, siglec-5, CD33, siglec-11, which can all down-regulate phagocyte activation and thereby contribute to tolerogenicity of the uptake process.

These results lead us to hypothesise that a selective loss of α2, 6-linked sialic acids contributes to an important set of signals on aged erythrocytes that allow physiological recognition and tolerogenic uptake by phagocytes.

P0266

LPS stimulation inhibits viral infection of human macrophages with two distinct mechanisms

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Purpose/Objective: It is well known that respiratory viral infections enable commensal bacteria in the nasopharynx to disseminate to other sites in the body and cause disease. However, this is not a uni-directional interaction and the presence of specific bacterial species in the nasopharynx may also affect viral pathogenesis. At present, it remains unclear if bacteria are able to exacerbate viral infections or if bacteria possibly serve to limit viral infections. We use influenza A virus (IAV) and respiratory syncytial virus (RSV) to examine how bacterial ligands in the nasopharynx can affect the pathogenesis of subsequent viral infections.

Materials and methods: Human monocyte derived macrophages were cultured from buffy coats and stimulated with bacterial ligands after which they were infected with GFP-IAV and GFP-RSV. Flow cytometry was used to determine percentage infection and phenotyping of the macrophages.

Results: Human epithelial cells (A549) stimulated by a panel of different bacterial ligands did not display any significant alteration in susceptibility to RSV or IAV. However, pre-stimulation of human monocyte derived macrophages with lipopolysaccharide (LPS) reduced the infection rate of both RSV and IAV with 80%. In contrast, bacterial ligands derived from Gram positive bacteria [lipoteichoic acid (LTA) and muramyl dipeptide (MDP)] did not inhibit viral infection. The protective effect was not due to pro-inflammatory cytokines or apoptosis of the macrophages. LPS protects against viral infection in two distinct mechanisms. It inhibited the entry of RSV early in the infection, whereas, in contrast, it triggered a long-term, non-specific, type I IFN response to prevent IAV infection.

Conclusions: Taken together, these data demonstrate the bi-directional nature of viral-bacterial interactions, and that the composition of an individual's nasopharyngeal flora may help determine their susceptibility to viral infections.

P0268

Maturation of distinct DC subsets is characterized by a profound and convergent genetic reprogramming

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Purpose/Objective: Dendritic cells (DC) express many innate immune recognition receptors able to sense a variety of microbial, danger or cytokine signals. The integration of these signals drives DC maturation and functional polarization during infection or tumorigenesis. Maturation is often assessed through the measurement of a few parameters, e.g. redistribution of MHC class II molecules from endosomes to plasma membrane, up-regulation of co-stimulation molecules and production of pro-inflammatory cytokines. Yet, DC maturation is much more complex and has been proposed to fundamentally differ between plasmacytoid DC (pDC) and conventional DC (cDC). To gain novel insights into this biological process, the gene expression reprogramming occurring in DC subsets upon activation was examined.

Materials and methods: Spleen DC subsets were isolated from untreated or murine cytomegalovirus (MCMV)-infected mice. Their gene expression was examined using microarrays. Gene chips data for other conditions of DC activation were generated in-house or retrieved from public repositories. Bioinformatics meta-analyses were performed. The expression pattern of selected genes was confirmed using custom-designed PCR-Arrays.

Results: In hierarchical clustering analysis, the 3 spleen DC subsets isolated from infected mice regrouped together, apart from their quiescent counterparts. This reflected that MCMV infection induced profound and convergent changes in the gene expression programs of all DC subsets *in vivo*. Indeed, while activated DC kept the transcriptional signature characteristic of their subset, they commonly induced and repressed several hundred of genes. Many genes followed the same pattern of regulation, whether the stimuli used were viruses or purified ligands for different Toll-like receptors, whether DC were

isolated *ex vivo* or derived *in vitro*, and whether the species studied were mouse or human.

Conclusions: This study allowed the identification of a core gene expression program associated with DC maturation irrespective of subsets, stimuli and species. DC maturation was associated with a remodeling of the gene expression program as profound and complex in cDC or monocyte-derived DC than in pDC. Maturation did not involve a true cell-fate conversion in any case since each activated DC subset kept a transcriptional blueprint of its specific ontogeny.

P0269

Mechanism of glucocorticoids action on monocytes during inflammation: reprogramming towards anti-inflammatory phenotype

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Purpose/Objective: We have previously demonstrated that the treatment of monocytes with GC does not suppress monocyte functions, but rather induces specific differentiation of cells with anti-inflammatory phenotype. Nevertheless the mechanism of GC action on monocytes during inflammation is currently not well defined. The aim of our studies was to investigate the effects of GC on pro-inflammatory monocytes.

Materials and methods: GC-, LPS- and GC-LPS-induced gene expression patterns in monocytes were analysed by microarray technology. The results were confirmed by quantitative RT-PCR. Protein expression and activation was analysed by Western Blot and Flow Cytometry. Monocyte functions essential for innate immunity migration, chemotaxis, phagocytosis, killing, oxidative burst were assessed.

Results: As expected, treatment of LPS-stimulated monocytes with GC resulted in inhibited expression of many of pro-inflammatory factors. Nevertheless, many of them have not been described to be regulated by GC in monocytes or any other cell type so far. Surprisingly, we have found that GC-LPS treatment of monocytes additionally led to synergistical up-regulation of many genes, which were not induced by GC or LPS alone. Moreover, we also observed additive effects of GC and LPS on expression of many anti-inflammatory genes which was much more pronounced compared to monocytes stimulated with GC alone. Analysis of the specific function of monocytes has shown that GC-LPS treatment inhibited specifically monocyte adherence, but enhanced spontaneous migration, chemotaxis, phagocytosis and killing of pathogens, and engulfment of apoptotic cells as well as the ability to produce anti-inflammatory lipid mediators.

Conclusions: Our results demonstrate that GC do not simply suppress LPS-mediated activation of monocytes, but rather induce their reprogramming toward a specific anti-inflammatory phenotype involved in resolution of inflammation.

P0271

Monocyte-derived tumour necrosis factor stimulated gene-6 (TSG-6) alters bone mass via effects on osteoblasts and osteoclasts

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Purpose/Objective: Osteoporosis and rheumatoid arthritis (RA) are associated with a disregulation of homeostatic bone turnover leading to bone loss and an increased risk of fracture. As inflammatory bone disease is costly to treat, and osteoporosis an increasing problem in an ageing population, there is an increasing need to develop anabolics for the promotion of localised bone formation.

Materials and methods: Skeletal health is likely dependent on the interaction of mesenchymal stem cells (MSCs) with those of the immune system. Using alkaline phosphatase levels and bone nodule formation assays, we have recently demonstrated that cell-cell interactions between monocytes and MSCs is necessary to drive the latter to differentiate into bone forming osteoblasts. To gain an insight into these monocyte-derived osteogenic factors, illumina whole genome microarray analysis was carried out after cellular contact. Of these, Tumour necrosis factor Stimulated Gene-6 (TSG-6) mRNA was found to be significantly up-regulated.

Results: TSG-6 is an ~ 30 KDa protein expressed in bone marrow under healthy physiological conditions, but has been found to accumulate in the synovial fluid of patients with inflammatory diseases such as RA. TSG-6 has been shown to have potent inhibitory effects on RANKL-mediated osteoclastogenesis, as well as BMP2-dependent alkaline phosphatase deposition, suggesting it is a bone homeostasis protein protecting against bone erosion and excess mineralisation respectively. These *in vitro* studies are unlikely to wholly reflect the role of this protein in the MSC niche, which is a source of many of the cellular precursors of bone remodelling and in which the interaction of all cell types with a protein determines its' function.

Conclusions: To further investigate the role of TSG-6 in bone homeostasis we are investigating i) the bone histomorphometry of WT and TSG-$6^{-/-}$ mice during ageing, ii) the effect of TSG-6 in *ex vivo* cultures and iii) the effect of inhibitors of known ligands of TSG-6 on *in vitro* assays.

P0272

Monocytes promote osteogenic differentiation of mesenchymal stem cells via PGE2 and Oncostatin M

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Purpose/Objective: Bone loss is a characteristic of chronic inflammatory and degenerative diseases such as rheumatoid arthritis and osteoporosis. A major challenge is how to replace bone once it is lost leading to the need for novel bone anabolic agents. The immune system regulates bone as demonstrated by the differentiation and activity of osteoclasts by immune cells and cytokines. However, less is known about the regulation of osteoblasts (OB).

Materials and methods: Mesenchymal stem cells (MSC) are multipotent progenitors that can be induced in culture to form OBs. Alkaline phosphatase (ALP) expression was assessed at 7 days and bone nodule formation at 28 days from MSC cultured in the normal and osteogenic media, in the presence or absence of human peripheral blood mononuclear cells.Cytokine levels were measured by ELISA and Luminex, microarray analysis used Illumina whole human genome chips.

Results: Monocytes/Mϕs potently induced MSC differentiation to OBs evidenced by increased ALP and mineralisation. The monocyte-mediated osteogenic effect was mediated by cell contact as well as monocyte-derived soluble factors.This was not due to BMPs or TGFβ production or cytokines such as IL-6, TNF and IL-10 that were found to be up-regulated following monocyte:MSC contact. Monocyte:MSC co-cultures also resulted in the up-regulation of PGE_2 whilst inhibition

of PGE_2 production by COX2 inhibitor NS-398 resulted in the abrogation of monocyte-induced osteogenesis. However, PGE_2 failed to directly induce osteogenic differentiation in MSC cultures indicating that it acts indirectly to induce the production of other factors by monocytes. Gene profiling microarray of monocytes identified Oncostatin M (OSM) as a mediator of monocyte-induced osteogenesis. The activation of STAT3 signalling by OSM in MSCs enhanced osteogenic differentiation; similar results were obtained using STAT3 constitutively active adenoviral overexpression.
Conclusions: This study establishes a role for monocyte/Mϕs as critical regulators of osteogenic differentiation via OSM production and the induction of STAT3 signalling in MSC. OSM, and other factors, induced by the contact between monocytes and MSC may be valuable new anabolic factors for use in osteoporosis and in localised bone remodelling during fracture and arthritis.

P0273

Mononuclear phagocytes produce VEGF that promotes neovascularization in laser-induced choroidal neovascularization

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Purpose/Objective: Age-related macular degeneration (AMD) is the most prevalent cause of blindness in the elderly, and its exudative subtype critically depends on local production of vascular endothelial growth factor (VEGF). In tumour models, mononuclear phagocytes (MPh) produce VEGF. MPh including dendritic cells, macrophages and microglia cells, express CX3CR1. MPh precursors, such as monocytes, can be recruited to sites of inflammation by CCR2. Here we studied VEGF production by MPh in a laser-induced murine model of choroidal neovascularisation (CNV) that mimics CNV in exudative AMD, and the impact of CCR2 on such production.
Materials and methods: CCR2-competent and -deficient CX3CR1-reporter mice expressing GFP in Mph were used to visualize these cells by 3 fluorescence-based techniques. CNV was induced by rupturing Bruch«s membrane beneath the retina by laser. 3 and 6 days later, eyes were enucleated and single cells from retina and choroid were stained for cellular subsets and for intracellular VEGF-production by flow cytometry. Neovascular areas were measured in choroidal and retinal flatmounts by isolectin staining. Scanning-Laser-Ophthalmoscopy (SLO) was used to reveal Mph in living animals.
Results: VEGF-positive phagocytes increased 3 days post laser, with microglia being dominant in the retina (twofold) and macrophages in the choroid (>25-fold). On day 6, VEGF-expressing microglia numbers remained increased, whereas macrophages had already declined. This temporary increase in macrophage numbers was abrogated in the absence of CCR2, whereas microglia was CCR2-independent. Consistent with flow-cytometry, microscopy and *in vivo SLO* revealed that Mph accumulated in CNV areas. This area was reduced in CCR2-deficient mice on day 14, but not later.
Conclusions: We describe an intracellular staining method for VEGF-production, a non-invasive *in vivo* imaging method for MPh in the eye, and identify Mph as relevant producers of VEGF in CNV. Infiltrating CCR2-dependent Mph only temporarily increase CNV, arguing against CCR2 inhibition as a successful solitary strategy to prevent AMD.

P0274

Nanomolar extracellular ATP promotes TLR4 signaling in dendritic cells to induce Th1 polarization through type I interferon

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Purpose/Objective: Dendritic cells (DC) can recognize both exogenous pathogen-associated molecules and endogenous dander signals. However, whether these stimuli can cooperate in functional regulation of DC has not been extensively investigated.
Materials and methods: Bone marrow-derived DC were treated with LPS (1 ng/ml) or recombinant HSP70 (1 μg/ml) in combination with different concentrations of extracellular ATP (1, 100, 100 and 1 mM). Then cytokine levels and DC phenotype were examined by ELISA and FACS, respectively. The activation of signaling pathways (MAPK, NF-κB and IRF3) were examined by either ELISA or Western blot. Finally, the effects of the *in vitro* induced DC on T cell proliferation and polarization were examined.
Results: We find that nanomolar extracellular ATP, released under nonfatal heat-stressed conditions, can synergize with Toll-like receptor (TLR)4 agonists, lipopolysaccharide (LPS) and heat shock protein (HSP)70, in promoting maturation and Th1-polarizing functions of DC. We demonstrate that nanomolar ATP promotes TLR4-triggered maturation of mouse bone marrow-derived DC and human monocyte-derived DC for Th1 response, in contrast to micromolar ATP-induced Th2-polarizing function of DC. In the presence of nanomolar ATP, TLR4-triggered interleukin (IL)-12p70 and type I interferon production as well as TRIF-dependent TBK1-IRF3 activation of DC are enhanced. In DC derived from $TLR4^{-/-}$, $IRF3^{-/-}$ or $IFNAR^{-/-}$ but not $MyD88^{-/-}$ mice, ATP fails to promote TLR4-induced type I interferon production, and subsequently IL-12p70 production or Th1-polarizing functions of DC. Additionally, we find that ATP-induced enhancement of TLR4 signaling relies on purinergic 2 (P2)Y receptor (P2YR)-mediated activation of phospholipase C-novel protein kinase C-Src tyrosine kinase signaling pathway.
Conclusions: Our study suggests that nanomolar extracellular ATP can cooperate with LPS or HSP70 in modulating DC functions for Th1 polarization potentially through TLR4-triggered type I interferon production. Our study thus possibly provides an explanation for efficient clearance of pathogens and induction of Th1 immune response under non-fatal infectious or injury conditions.

P0275

NK-DC interaction is controlled by an activin A-dependent negative feedback pathway

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Purpose/Objective: Crosstalk of natural killer (NK) cells with dendritic cells (DC) leads to reciprocal activation. Although some of the mechanisms involved in this interaction have been described, the characterization of the molecules involved deserves further attention.
Materials and methods: A PCR array-based gene expression screen, focused on common cytokines, was performed on human monocyte-derived DC cocultured with allogenic NK cells, pre-activated with IL-

15. The array led to the identification of activin A in NK-DC co-cultures. In order to determine the functional role of endogenously produced activin A during NK-DC interaction we analyzed DC maturation (CD83 and CD86 expression) and the release of cytokines (i.e. IL-1β, IL-6, IL-8, IFN-γ, TNF-α and GM-CSF) using follistatin, a natural inhibitory protein of activin A.
Results: Among a set of 84 cytokines investigated, activin A is the second highest induced cytokine in NK-DC co-cultures, with IL-8 being the most upregulated gene. Activin A is a member of the TGF-b superfamily that was previously shown to possess both pro- and anti-inflammatory activities and to control certain DC and NK cell functions. In NK-DC co-culture experiments, the induction of activin A required cell contact and was dependent on the presence of pro-inflammatory cytokines (i.e. IL-1β, IFN-γ, TNF-α and GM-CSF) and cell death. Conversely, the expression of activin A was negatively controlled by HLA-specific inhibitory receptors. In co-culture experiments, inhibition of acitivn A by follistatin resulted in the upregulation of pro-inflammatory cytokine release (i.e. IL-6, IL-8, TNF-α) and in the increased NK cell-induced DC maturation, as evaluated by CD83 and CD86 expression.
Conclusions: In conclusion, our study suggests that the production of activin A during NK-DC interaction represents a relevant negative feedback mechanism to prevent excessive immune activation.

P0276
NR4A1 mediates anti-inflammatory effects of apoptotic cells

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Purpose/Objective: The nuclear receptor NR4A1 has been implicated as negative feedback regulator of NF kappa B signalling and as key regulator during the differentiation of Ly6C-low resident monocytes. Apoptotic cells are known to exert anti-inflammatory effects on macrophages but the underlying mechanisms are still poorly understood. Here we studied a potential role of NR4A1 as mediator of the macrophage response to apoptotic cells.
Materials and methods: We analysed the effect of apoptotic thymocytes on wild type and NR4A1$^{-/-}$ peritoneal resident macrophages, and determined the consequences on intracellular signalling, gene expression and cytokine profile. Moreover, we examined the consequences of the lack of NR4A1 during maintenance of self tolerance by using the pristine-induced model of murine sytemic lupus erythematosus.
Results: Expression of NR4A1 was rapidly and highly induced in resident macrophages after incubation with apoptotic thymocytes. NR4A1$^{-/-}$ resident macrophages showed an exacerbated pro-inflammatory profile as well as an increased activity of NF-κB. Moreover, the anti-inflammatory effects of apoptotic cells were reduced in NR4A1$^{-/-}$ macrophages. In the pristine model of murine lupus, NR4A1$^{-/-}$ mice displayed increased levels of autoantibodies such as ds-DNA antibodies.
Conclusions: Tacking together, this data show for the first time that NR4A1 is an important mediator of the anti-inflammatory effects of apoptotic cells in tissue resident macrophages and thereby contributes to the maintenance of self-tolerance.

P0277
Osteoactivin is produced by tumor-conditioned monocytes/macrophages

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Purpose/Objective: Tumor-associated macrophages (TAM) are key orchestrators of the tumor microenvironment directly affecting neoplastic cell growth, neoangiogenesis, and extracellular matrix remodeling. In a gene profiling analysis of human monocytes co-cultured with M-CSF-expressing tumor cells, we identified a number of up-regulated genes. One of the most expressed gene was Human Glycoprotein non-metastatic melanomal protein B (GPNMB) also called Osteoactivin or Mouse Dendritic cell-associated Heparan Sulfate Proteoglycan-Integrin Ligand (DC-HIL). Osteoactivin is a transmembrane molecule with diverse biological functions spanning from cell adhesion and migration, to immune-suppression and tissue repair. This study investigates the modulation of Osteoactivin and its functional role inmonocytes/macrophages and TAM.
Materials and methods: Monocytes and macrophages were tested for expression and modulation of Osteoactivin after treatment with different stimuli in RT-PCR, flow cytometry and ELISA. *In vivo* mouse models of tissue injury and tumor growth in mice bearing a spontaneous mutation of Osteoactivin (DBA2J strain).
Results: In macrophage/tumor cell co-cultures the protein Osteoactivin was mainly produced by macrophages and not by tumor cells. Among the different stimuli tested, the strongest up-regulation was observed with M-CSF, Corticosteroids and TGFb. In contrast GM-CSF, IL-1 or TNF were poor stimulators. Osteoactivin expression by *in vitro* polarized macrophages was higher in IL-4-stimulated M2 macrophages and further increased in hypoxic conditions. Prolonged *in vitro* culture of monocytes/macrophages induced cleavage of Osteoactivin from cell membranes (not before 4 days). Osteoactivin had no effect on myeloid cell differentiation or tumor cell proliferation, but did enhance monocyte adhesion via its integrin binding domain. MutantOsteoactivin mice (*DBA/2J*) andmice reconstituted with the wild type gene (*DBA/2J-Gpnmb*$^{+}$) are currently being studied in mouse tumor and tissue injury models.
Conclusions: Osteoactivin is an interesting moleculeproduced by tumor-conditioned and M2-polarizedmacrophages and modulated by M-CSF, corticosteroids and hypoxia. Its role in the injured tissues or tumor-micro-environment is as yet not clarified.

P0278
Paired immunoglobulin-like receptor B (PIR-B) negatively regulates pulmonary fibrosis by suppressing IL-4-induced macrophage activation

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Purpose/Objective: Macrophages are pleotropic cells, which possess fundamental roles in innate and adaptive immunity. Recent data highlight that a central IL-4: macrophage axis exists in fibrotic settings since IL-4-activated macrophages (i.e. M2 macrophages) express numerous pro-fibrogenic mediators and can promote tissue repair and remodeling. Paired immunoglobulin-like receptor B (PIR-B) is a prototype inhibitory receptor capable to counter-regulate macrophage activation. However, the expression and function of PIR-B in the regulation of M2 macrophage responses and pulmonary fibrosis is unknown.

Materials and methods: Wild type (WT) and *Pirb*$^{-/-}$ mice were intratrachealy-challenged with bleomycin (BLM) or recombinant IL-4. PIR-B expression in the lungs of WT mice was assessed (qPCR, flow cytometry). Bronchoalveolar lavage following BLM or IL-4 administration was assessed for differential cell counts as well as cytokine and chemokine expression (ELISA). Bone marrow (BM)-derived WT and *Pirb*$^{-/-}$ macrophages were stimulated with IL-4 and assessed for various M2-associated molecules.
Results: PIR-B was upregulated in several lung myeloid cell subsets (CD45^{+} /CD11c^{high}/Gr-1^{high} and CD45 /CD11c^{high}/Gr-1^{int}) following BLM administration. BLM-treated *Pirb*$^{-/-}$ mice evidenced significantly increased lung fibrosis in comparison with BLM-treated WT mice (e.g. increased lung histopathology, collagen content and α-smooth muscle actin expression). Increased disease severity was accompanied with increased lung levels of resistin-like molecule α (Relm-α), matrix metalloproteinase 12 (MMP-12), tissue inhibitor of metalloproteinase 1 (TIMP1) and osteopontin (OPN). Notably, flow cytometric analysis revealed increased Relm-α^{+} and MMP-12^{+} alveolar macrophages in BLM-treated *Pirb*$^{-/-}$- mice. Consistently, administration of IL-4 to WT and *Pirb*$^{-/-}$ mice resulted in increased total lung Relm-α expression and Relm-α^{+}/MMP-12^{+} alveolar macrophages in *Pirb*$^{-/-}$ mice. Furthermore, IL-4-activated BM-derived *Pirb*$^{-/-}$ macrophages displayed increased Relm-α, MMP12 and TIMP-1 induction in comparison with wild type cells. Finally, PIR-B human orthologues (e.g. ILT-5/CD85a) were readily detected in lung biopsies obtained from idiopathic pulmonary fibrosis patients (IPF).
Conclusions: Our results establish a key role for PIR-B in pulmonary fibrosis by negatively regulating IL-4-induced macrophage activation and suggest a possible role for PIR-B human orthologues in IPF.

P0279
PHD inhibition induces M2 macrophage polarisation and has differential effects on lung fibrosis

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Purpose/Objective: Pulmonary fibrosis is a common consequence of lung inflammation, leading to organ dysfunction and hypoxia. Prolyl hydroxylases (PHDs) are the main oxygen sensors and regulators of hypoxia inducible factors (HIFs) in cells and thus key enzymes involved in hypoxic signalling. Several studies have shown that manipulation of hypoxic or HIF signalling in tissues and specifically macrophages can promote tissue healing. However, macrophages are also recognised to contribute to fibrosis.
Materials and methods: We aimed to investigate the effect of PHD inhibition in bleomycin induced murine lung injury and its effect on macrophage polarisation. We hypothesised that PHD inhibition may induce alternatively activated (M2) macrophages that may have pro-resolution or pro-fibrotic potential dependent upon their temporal expression. For this purpose, we used the bleomycin model which exhibits two phases, an early inflammatory phase followed by a late fibrotic phase.
Results: Pharmacological PHD inhibition was achieved using DMOG and FG0041. DMOG was administered in both the early and the late phase of the bleomycin model. End points included lung collagen content and measures of lung inflammation. Administration of DMOG during the early, inflammatory phase of the model significantly reduced lung fibrosis. In contrast, late phase administration enhanced lung fibrosis. DMOG given in the early phase does not reduce lung inflammation or inhibit collagen synthesis, but there was evidence of enhanced M2 macrophage polarisation. *In vitro*, both DMOG and FG0041 enhanced M2 macrophage activation. Genetic manipulation using LysM/Cre HIF2A KO mice showed that M2 macrophage enhancement by PHD inhibition is HIF2A independent.
Conclusions: We have demonstrated that PHD inhibition induces M2 macrophage activation independent of HIF2A. This may be a potential therapeutic strategy in lung fibrosis.

P0280
Phenotype and localization of a novel subpopulation of IFNß producing plasmacytoid dendritic cells *in vivo*

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Purpose/Objective: During the initial response to viral infections type I interferons (IFN) are crucial mediators of subsequent protective immune mechanisms. It is well established that plasmacytoid dendritic cells (pDCs) are potent producers of type I IFN. Recently we could show that IFNβ is produced mainly by pDCs after TLR9 stimulation with CpG *in vivo* using an IFNβ/YFP reporter-knockin mouse model (IFN$\beta^{mob/mob}$). These IFNβ producing pDCs are located in the spleen within the T cell zone of the white pulp, whereas according to published data pDCs are located within the marginal zone. We suggest that these pDCs define a unique subpopulation and due to the increasing variety of pDC surface-markers we performed a comprehensive study on phenotype and localization of these up to now ill-defined IFN producing pDCs.
Materials and methods: C57BL/6, IFN$\beta^{mob/mob}$, IFNAR$^{-/-}$ or IFNAR$^{-/-}$xIFN$\beta^{mob/mob}$ mice were i.v. injected with CpG 1668 complexed to DOTAP or left untreated and organs were harvested after 6 h. For FACS analysis cells were stained for CD3ε, CD19, CD11c, mPDCA-1, B220, SiglecH, CCR9 and CD9. For histology spleen sections were fixed with acetone or paraformaldehyde followed by a staining for B220, CD11c, mPDCA-1, SiglecH or YFP and visualized by fluorescence microscopy.
Results: Our FACS data indicate that only a minor fraction of pDCs that are CD11c^{int}B220^{+} mPDCA-1^{+} SiglecH^{+}CCR9^{+} CD9^{-} produce IFNβ early (6 h) after TLR9 stimulation. Extending our previous histological findings YFP/IFNβ^{+} cells are co-positive for pDC markers and can be found at the interface of the T and B cell areas of the spleen whereas staining with different pDC markers like mPDCA-1 or SiglecH shows the vast majority of pDCs in the areas around the marginal zone.
Conclusions: Thus we assume that IFNβ producing cells constitute a unique subpopulation within the classical CCR9^{+} B220^{+} pDCs. IFNβ producing pDCs show a unique localization within the spleen *in vivo*. This is complemented by a microarray based transcriptome analysis where we found more than 1500 genes differentially expressed between IFNβ-producing and non-producing pDCs after TLR9 stimulation. Further analyses of this pDC subpopulation at additional time points after stimulation and the impact of the IFN type I receptor mediated feedback loop are currently under way.

P0282
Porphyromonas gingivalis LPS induces cytokine secretion in macrophages without costimulatory marker expression

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Purpose/Objective: Ligation of Toll-like receptors (TLRs) on macrophages by bacterial pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) leads to macrophage maturation, pathogen phagocytosis, antigen presentation and recruitment of immune effector cells. Macrophages primed with interferon gamma (IFN-γ) and exposed to PAMPs mature into classically acti-

vated macrophages (M1 macrophages). M1 macrophages synthesise high levels of microbicidal compounds and costimulatory molecules and are involved in pathogen clearance. *Porphyromonas gingivalis* is a key pathogen associated with chronic periodontitis. Mono-phosphorylated tetra-acylated or penta-acylated LPS from *P. gingivalis* has been shown to induce variable immune responses in mammalian cells when compared to other gram negative pathogens. However the effect of priming macrophages with cytokines prior to TLR ligation has yet to be investigated.

Objective: To investigate the effect of *P. gingivalis* LPS on the maturation and function of M1 macrophages.

Materials and methods: Mouse bone marrow-derived macrophages were primed with IFN-γ then exposed to *Escherichia coli* LPS, the synthetic lipoprotein PAM3Cys or *P. gingivalis* LPS. Macrophage maturation was then monitored by analysis of costimulatory markers and the production of nitric oxide and various inflammatory cytokines.

Results: Macrophage primed with IFN-γ and activated with 10 ng/ml of *E. coli* LPS or PAM3Cys resulted in a significant upregulation of costimulatory molecules and the production of nitric oxide and inflammatory cytokines, indicating classical activation. However activation of macrophages with *P. gingivalis* LPS did not, even at concentrations 100-fold higher. Despite the lack of costimulatory marker expression, M1 macrophages stimulated with 10 μg/ml *P. gingivalis* LPS produced significant levels of the cytokines TNF-α, IL-6, IL-1β and IL-12.

Conclusions: Our results demonstrated that M1 macrophages activated with *P. gingivalis* LPS were unable to fully differentiate into a classically activated macrophages, even at high concentrations of LPS. These results suggest that the impaired ability of macrophages to recruit cells of the adaptive immune system and kill bacteria may contribute to the inability to clear *P. gingivalis* during chronic periodontitis.

P0283
Presence of CCR7-independent transport of skin antigens from the dermis

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Purpose/Objective: The transport of peripheral self-antigens (self-Ags) to regional lymph nodes (LNs) is thought to play a critical role in the maintenance of peripheral tolerance. Dendritic cells (DCs) play a major role in the capture and transport self-Ags. It has been reported that a chemokine receptor CCR7 dominantly regulates the migration of skin DCs to regional LNs in a steady state. However, CCR7-knockout (*Ccr7*$^{-/-}$) mice do not show any skin autoimmune disorders. To determine whether the transport of skin self-Ags is actually impaired under CCR7-deficient conditions, we examined *Ccr7*$^{-/-}$ mice.

Materials and methods: The transport of skin self-Ags to regional LNs in *Ccr7*$^{-/-}$ mice was examined using epidermis- or dermis-hyperpigmented transgenic mice.

Results: While the transport of epidermal self-Ags was considerably disturbed in 7-week-old *Ccr7*$^{-/-}$ mice, the transport was clearly observed in 30-week-old *Ccr7*$^{-/-}$ mice. In these aged mice, the accumulation of epidermal self-Ags in the dermis was also observed. Next we investigated the transport of dermal self-Ags in 7-week-old *Ccr7*$^{-/-}$ mice, and found the transport of dermal self-Ags to regional LNs. Immunohistochemistry and cell sorting indicated the involvement of CD11c^{+} cells in this transport, while CD205^{+} cells, CD207^{+} cells and $\gamma\delta$ T cells might not significantly contribute to it.

Conclusions: Skin self-Ags were transported under CCR7-deficient conditions. This CCR7-independent system is present in the dermis, and DC-lineage cells other than CD207^{+} dermal DCs or CD205^{+} Langerhans cell-descendants should contribute to this transport. The dermal system might support the continuous transport of both epidermal and dermal self-Ags even under CCR7-deficient conditions.

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P0284
Protective role of antioxidant systems in human monocytic cell lines during differentiation and stimulation with a TLR2 ligand

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Purpose/Objective: Monocytes and macrophages recognize and kill pathogens during many processes resulting in production of a number of oxygen-containing compounds. The aim of this study was to investigate changes in antioxidant defense status of this cell lineage during differentiation and exposition to pathogen associated molecular patterns. THP-1 and Mono Mac 6 (MM6), human monocytic cell lines, can serve as an appropriate model to test susceptibility of undifferentiated and differentiated (macrophage like) cells to oxidative conditions.

Materials and methods: Cell lines were differentiated for 72 h: THP-1 with PMA, MM6 with Vitamin D3 and TGF-β. For TLR2 activation, cells were stimulated with Pam3CSK4. Gene expression levels were assessed by real time PCR. Activity of redox protective enzymes and glutathione content were measured biochemically. Susceptibility of cells to H_2O_2 was tested using a resazurin-based assay.

Results: Glutathione level was comparable in THP-1 and MM6 and this concentration increased in THP-1 as a result of differentiation. The basal level of antioxidant enzymatic activity in MM6 was higher than in THP-1, but in THP-1 most of it increased upon differentiation up to the level seen in MM6. Similar results were seen with expression level of genes encoding main antioxidant enzymes: in MM6, the basal level of expression of these genes was higher; differentiation and TLR2 stimulation caused gene activation apart from the thioredoxin system in MM6, which was not up-regulated.

Pretreatment of THP-1 with Pam3CSK4 as well as differentiation caused these cells to become more resistant to H_2O_2-mediated cell death. On the other hand, low susceptibility to H_2O_2 was characteristic for MM6 and this high resistance was not further modulated either upon Pam3CSK4 treatment or upon differentiation.

Conclusions: In summary, MM6 cells have a high level of crucial antioxidant enzymes conferring a strong resistance to H_2O_2 in the basal state and this resistance is largely unmodified upon differentiation or stimulation. THP-1 cells are more susceptible to oxidative stress, but TLR2 stimulation or differentiation both result in significant induction of redox protecting enzymes, especially thioredoxin reductase. Thus, THP-1 cells are probably well-suited for studies on the role of the thioredoxin system in oxidative stress response in macrophages.

P0285
Proteinase activated receptor-2 (PAR-2) modulation of human macrophage subset differentiation and effector function

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Purpose/Objective: Proteinase activated receptor-2 (PAR-2) is a G protein coupled receptor expressed by a variety of cell types, including

macrophages. Activated by specific serine proteases, an emerging role for PAR-2 in immune regulation has been recognized. Despite advances in the understanding of PAR-2 biology, its role in human macrophage subset development and effector function remains relatively unexplored. The aim of this study was to elucidate the role of PAR-2 in GM-CSF and M-CSF derived macrophages.

Materials and methods: $CD14^+$ monocytes purified from human peripheral blood were cultured for 6 days with GM-CSF or M-CSF. Matured cells were activated with a PAR-2 activating peptide (SLIG) and regulation of PAR-2 expression assessed by FACS while TNFα and IL-10 levels were measured by ELISA.

In differentiation experiments, monocytes were matured with GM-CSF or M-CSF ± SLIG for 6 days followed by LPS stimulation for 48 h and supernatants collected for cytokine analysis.Cell area was measured using Zeiss Axiovision LE software to assess morphological changes.

Results: Both GM-CSF and M-CSF derived macrophages expressed PAR-2 which was up regulated by SLIG. GM-CSF cells activated with SLIG made significantly more TNFα than M-CSF cells [9740 ± 152 pg/ml versus 3487 ± 309 pg/ml, $P < 0.001$], and significantly less IL-10 [27 ± 3 pg/ml versus 769 ± 73 pg/ml, $P < 0.001$].

Macrophage subsets polarized in the presence of SLIG had an altered morphology, with a significant reduction in cell area ($P < 0.05$). GM-CSF cells matured with SLIG produced significantly less TNFα when LPS challenged compared to control cells (6043 ± 793 pg/ml versus 9230 ± 765 pg/ml, $P < 0.05$), with no significant change in IL-10. In contrast, M-CSF cells matured with SLIG produced significantly more TNFα compared to control cells (5730 ± 187 pg/ml versus 3390 ± 748 pg/ml, $P < 0.05$) with reduced IL-10 secretion (11 ± 3 pg/ml versus 2062 ± 472 pg/ml, $P < 0.01$).

Conclusions: PAR-2 activation in macrophage subsets up regulates PAR-2 expression and drives the release of signature cytokines. Activation of PAR-2 during macrophage differentiation appears to skew cell phenotype, as assessed by cytokine profiles. This may be relevant in macrophage driven disease where protease rich environments may influence macrophage effector function.

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P0286

Regulatory activity of DC-10 IS stringently dependent on the levels of hla-g expression

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Purpose/Objective: DC-10 are a subset of human tolerogenic DC which efficiently prime T cells to become adaptive IL-10-producing type 1 regulatory (Tr1) cells. DC-10 secrete high levels of IL-10 and strongly express the tolerogenic molecules Immunoglobulin-Like-Transcript (ILT)4 and HLA-G, which are critically involved in the differentiation of Tr1 cells *in* vitro. Although all these three players, ILT4, HLA-G and IL-10 are important for DC-10 regulatory activity, it still remains to define their relative contribution in promoting tolerance *via* Tr1-cell induction. To dissect the role of HLA-G in tolerance associated with DC-10, we took the advantage that polymorphisms at 3' untraslated region (UTR) of the HLA-G gene locus might influence its expression.

Materials and methods: Healthy donors have been typed for polymorphisms at the 3' UTR of HLA-G locus and classified according to the presence of 14bp Insertion/Deletion (Ins/Del) and C/G in position$^+$3142 as following: DelC/DelC, InsG/InsG, DelG/X, and DelC/InsG. DC-10 were differentiated from 3'UTR HLA-G typed donors, by culturing $CD14^+$ monocytes in the presence of IL-4, GM-CSF and IL-10 for 7 days, and then were phenotypically and functionally characterized.

Results: Results demonstrated that DC-10 derived from DelC/DelC or DelG/X monocytes express significantly higher percentage of HLA-G as compared to DC-10 derived from InsG/InsG monocytes. DC-10 independently from 3'UTR polymorphisms poorly stimulate allogeneic T cells. Interestingly, DC-10 expressing high levels of HLA-G induced alloantigen-specific anergic T cells, whereas T cells stimulated with DC-10 expressing low levels of HLA-G display significant proliferation. This effect is independent from both ILT4 and IL-10, since DC-10 from different 3'UTR HLA-G typed donors expressed comparable levels of ILT4 and secrete similar amounts of IL-10.

Conclusions: In conclusion our findings demonstrate that the expression of HLA-G on DC-10 is critically required for promoting anergy in T cells and Tr1 cells. These results represent the first step forward to understand how DC-10 promote/restore tolerance *in vivo* and provide us with new tools for tailoring protocols for *in vitro* generation of antigen-specific Tr1 cells suitable for cell therapy *via* tolerogenic DC-10.

P0287

Role of UNC93B1, a chaperone molecule, in the MHC class I cross presentation pathway

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Purpose/Objective: Several studies have suggested that intracellular TLRs can be targeted directly from the endoplasmic reticulum (ER), where they reside, to endosomes in which they signal. Mouse and human genetics have identified UNC93B1, which encodes for a highly conserved 12-membrane spanning molecule residing in the ER, as a key regulator in the transport of intracellular TLRs (7, 8 and 9). The 3d mutation (UNC93B1 mutation) results in a phenotype where no signalling occurs via the intracellular TLRs 3, 7 and 9 and also diminishes presentation of exogenous antigen. However, the mechanism that lies beneath and the exact role that UNC93B1 displays on antigen presentation remain to be fully elucidated.

Materials and methods: All our experiments *in vitro* were performed in primary dendritic cells (GM-CSF derived and $CD8^+$ cDCs purified from the spleen). We used a panel of many different tecnics *in vitro* (siRNA, transfection, confocal microscopy, ELISA) and *in vivo* (i.v antigen injection and T cell proliferation using CFSE dilution).

Results: Firstly, we show that MHC I cross presentation of exogenous antigen is drastically impaired in UNC93B1-mutated $CD8^+$ and inflammatory dendritic cells *in vitro* as well as in UNC93B1 mutated mice (3d mice) *in vivo*. Phagosomes are the dedicated compartments for antigen cross presentation. Our data show that the fusion with late endosomal compartments during phagosomal maturation is increased in UNC93B1-mutated DCs suggesting that ER-phagosome interaction may be altered. Moreover, UNC93B1 mutation in dendritic cells remarkably reduced antigen degradation and export of processed antigens into the cytoplasm. Notably, UNC93B1 deficiency impairs endosomal/phagosomal acidification and decreases the proteolytic activity in phagosomes. Altogether, these data strongly suggest an 'abnormal' phagosomal environment in UNC93B1-mutated DCs leading to severely reduced MHC class I cross-presentation. Finally, immunoprecipitation and immunofluorescence assays reveal new binding partners for UNC93B1 that display a critical role on antigen presentation.

Conclusions: Our findings provide new insights into the mechanism employed by UNC93B1, a chaperone molecule, to hijack the MHC I cross presentation pathway.

P0289

SOCS3 promotes M1 macrophage activation and function through effects on nucler factor kappaB activity

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Purpose/Objective: Macrophages respond to their microenvironment and develop polarised functions critical for orchestrating an appropriate inflammatory response. M1-activated macrophages eliminate pathogens, but can cause host tissue damage, while M2-activated macrophages promote regulation and repair. M1-macrophage activation is strongly associated with SOCS3 expression but the functional consequences of this, especially *in vivo*, are not clear. The aims were to determine the phenotype and function of SOCS3-expressing macrophages in immune-mediated glomerulonephritis, and address mechanisms by which SOCS3 controls macrophage pro-inflammatory potential.
Materials and methods: Triple immunohistochemistry determined the number of CD68 positive macrophages co-expressing SOCS proteins and the M1-activation marker iNOS in kidney sections of animals with experimental nephritis; severity of nephritis was assessed by albuminuria. SOCS3 was knocked down in bone marrow derived macrophages by specific siRNA and NFκB-p65 activity determined by EMSA based ELISA. Western blotting defined IκB-α degradation and PI3K activation.
Results: Macrophages infiltrating inflamed glomeruli show up-regulation of SOCS3 that co-localises with iNOS. The correlation between the number of SOCS3 (but not SOCS1) expressing glomerular macrophages and severity of nephritis was highly significant supporting their pro-inflammatory role in *vivo*. siRNA mediated knockdown of SOCS3 in macrophages adoptively transferred and conditioned in the inflamed peritoneum demonstrate its importance in driving production of pro-inflammatory mediators IL-6 and nitric oxide, and curtailing expression of anti-inflammatory IL-10 and SOCS1. The SOCS3 induced pro-inflammatory effects were due, at least in part, to its role in controlling NFκB nuclear activity. Surprisingly, SOCS3 also limits the activity of PI3K that is thought to regulate NFκB activation and using PI3K inhibitors we reveal that this may provide a novel mechanism by which SOCS3 controls macrophage pro-inflammatory properties.
Conclusions: These data demonstrate the importance of SOCS3 in directing and maintaining macrophage activation to a pro-inflammatory phenotype and emphasise that decreasing macrophage SOCS3 expression has potential as a therapeutic manoeuvre to limit autoimmune and inflammatory diseases.

P0290

Subsets of alternatively activated human macrophages show differential capacity to produce reactive oxygen species

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Purpose/Objective: We recently showed that ROS production by type 2 macrophages (Mph2) is involved in immunosuppressive properties and in the induction of regulatory T cells. Mph display a high plasticity depending on the cytokine milieu, and various Mph2 subsets with different functional characteristics have been described. In the current study we investigated ROS producing capacity of well defined human Mph subsets and studied the contribution of ROS in the APC-T cell interaction.
Materials and methods: Monocytes were differentiated into macrophages with M-CSF (Mph2), IL-4 (Mph2a), or IL-10 (Mph2c). The ROS producing capacity of these Mph was tested by flow cytometry using DHR123, and expression of the NADPH oxidase (NOX2) complex was analyzed at mRNA and protein level. T cell stimulatory capacity of the Mph was investigated by allogeneic MLR. The effect of DPI, an inhibitor of the NOX2 complex, was investigated both in MLR as well as directly on Mph regarding MHC and CD86 expression, and cytokine production.
Results: Upon PMA stimulation, Mph2 and Mph2c showed a high ROS producing capacity, whereas only low ROS production was observed in Mph2a. Mph2a expresses lower mRNA and protein levels of NOX2 protein $gp91^{phox}$ compared to Mph2 and Mph2c, whereas no differences were observed for $p47^{phox}$. Mph2 and Mph2c displayed a reduced T cell stimulatory capacity compared to Mph2a. However, we were not able to restore the T cell stimulatory capacity by the use of the NOX2 inhibitor DPI. In contrast, DPI even decreased the T cell proliferation in a MLR in concentrations that did not affect CD3/CD28-activated T cells. When tested directly on Mph2 subsets, levels of HLA-DR and CD86 levels were not affected by DPI. However, DPI specifically decreased the LPS-induced and CD40L-induced IL-10 and IL-12p40 production of all Mph2 subsets.
Conclusions: The ROS producing capacity is not the same for different anti-inflammatory Mph subsets; Mph2 and Mph2c have a higher ROS producing capacity compared to Mph2a, possibly related to a reduced $gp91^{phox}$ expression of the latter. The difference in T cell stimulatory capacity between Mph2 subsets is not directly linked with this differential ROS producing capacity. In contrast, DPI inhibited T cell stimulation and cytokine production by all Mph2 subsets, indicating a ROS-dependent mechanism of T cell activation.

P0291

Suppressors of cytokine signaling 2 and 3 diametrically control macrophage polarisation

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Purpose/Objective: Suppressors of cytokine signalling (SOCS) are important regulators of LPS and cytokine responses but their role in the control and regulation macrophage polarisation is unknown.
Materials and methods: We investigated the role of SOCS proteins using *in vivo* knockout mouse models of endotoxic shock, polymicrobial sepsis (Caecal ligation and puncture) and adoptive transfer. *In vitro* and *ex.vivo* we underscored the mechanistic actions of these proteins using techniques such as Q-PCR, Chromatin Immunoprecipitation, ELISA, Western blot and Flow cytometry.
Results: Myeloid restricted SOCS3 deletion ($SOCS3^{LysMcre}$) resulted in profound resistance to endotoxic shock, whereas on the other hand $SOCS2^{-/-}$ mice were highly susceptible. We observed a striking bias towards M2-like macrophages in $SOCS3^{LysMcre}$ mice, whereas in $SOCS2^{-/-}$ the M1-like population was enriched. Through adoptive transfer experiments we show that these antipodal responses to endotoxic shock and to polymicrobial sepsis were both transferable and entirely macrophage-dependent. Critically this dichotomous response was associated with enhanced Treg recruitment by the $SOCS3^{-/-}$ cells, but in the presence of $SOCS2^{-/-}$ macrophages $Foxp3^{+}$ T cells were completely absent at the inflammatory site. The altered polarisation coincided with enhanced IFNg-induced STAT1 in $SOCS2^{-/-}$ macrophages and enhanced IL-4/IL-13 induced STAT6 phosphorylation in $SOCS3^{-/-}$ cells.

Conclusions: Therefore SOCS are essential controllers of macrophage polarisation and thereby regulate the inflammatory response.

P0292

Tetraspanin CD82 plays a vital role in antigen presentation and processing in dendritic cells, and influences migration in opposition to CD37

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Purpose/Objective: Tetraspanins are a superfamily of membrane molecules which form microdomains to organise the cell surface. CD82 is a broadly expressed molecule best characterized as a metastasis suppressor gene, and is the closest phylogenic relative of the tetraspanin CD37. CD82 is strongly linked to homotypic and heterotypic cell-cell adhesion and CD37 is known to negatively regulate MHC/peptide presentation. Here we examine the affect of CD82 ablation using a new knockout mouse model.
Materials and methods: CD37KO DCs are hyperstimulatory to T cells *in vitro*, and this effect is due to an increase in MHC-peptide presentation, as measured by interaction of peptide pulse DCs with hybridoma cells. We use this cellular assay and others based on the OT1/OT2 antigen model to examine CD82KO DCs. We also look at the affect of *in vitro* differing DC morphology on DC migration both *in vitro* and *in vivo* using intravital microscopy and inflammatory models.
Results: CD82 ablation causes a significant decease in the ability of dendritic cells to stimulate T cell proliferation *in vitro*. We show that this is not due to uptake, surface expression, costimulation or MHC-peptide presentation, but due to a decrease of conjugate formation between DCs and T cells, possibly linked to a failure of formation of dendrites in mature DCs. We show that CD82 and CD37 appear to act in opposition in migration of DCs, with CD82 loss increasing migration potential, while loss of CD37 inhibits this process.
Conclusions: Our data indicates that CD82 and CD37 act in different mechanisms in antigen presentation and act in opposition in migration. CD82 acts on the physical interaction between DCs and T cells, and may act upon the same mechanism to influence DC migration *in vivo*. Our results have implications in the role of CD82 and its involvement in cellular migration, as seen in metastasized cancer.

P0293

The $CD14^{++}/CD16^{+}$ monocyte subset promotes the expansion of Th17 cells in rheumatoid arthritis

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Purpose/Objective: Monocytes can be subdivided by their expression of CD14 and CD16. The 'classic' monocyte subset expresses high levels of CD14 and no CD16 ($CD14^{++}/CD16^{-}$), the 'non-classical' monocytes express low levels of CD14 and CD16 ($CD14^{+}/CD16^{+}$) and the third subset, described as pro-inflammatory, is classified by their high expression of CD14 and CD16 ($CD14^{++}/CD16^{+}$). Aim of this study was to analyze the monocyte subpopulations in patients with rheumatoid arthritis (RA) and to characterize the $CD14^{++}/CD16^{+}$ monocyte subpopulation.
Materials and methods: Human PBMCs were isolated by density gradient centrifugation. Monocytes were enriched by depletion of other cells using antibody-coupled microbeads. Subsequently, monocyte subpopulations were sorted according to their CD14 and CD16 expression by flow cytometry and cocultured with $CD4^{+}$ T cells in the presence of LPS for 5 days. After 12 days Th17 cells were detected by flow cytometry. Cytokines were measured in the supernatant by ELISA.
Results: The analysis of the monocyte subpopulations in patients with RA compared to healthy controls shows a higher frequency of $CD14^{++}/CD16^{+}$ monocytes in patients with RA (median 10.1%, $n = 60$ versus median 5.4%, $n = 30$, $P < 0.0001$).

$CD14^{++}/CD16^{+}$ monocytes produce more pro-inflammatory cytokines (IL-1β, IL-1α and TNF) compared to $CD14^{+}/CD16^{+}$ and $CD14^{++}$/CD16- subsets. The co-culture of LPS-activated monocyte subsets with $CD4^{+}$ T cells led to the expansion of Th17 cells. In the presence of $CD14^{++}/CD16^{+}$ monocytes, more Th17 cells were expanded than in co-cultures with the two other monocyte subpopulations. Additionally, more IL-23 and IL-1β was detected in co-cultures of $CD14^{++}/CD16^{+}$ monocytes with $CD4^{+}$ T cells in comparison to the other monocyte subpopulations.

ex vivo analysis of the blood from RA patients and healthy donors indicate an increase of Th17 cells in patients with RA. This increased frequency correlates strongly with the higher frequency of $CD14^{++}/CD16^{+}$ monocytes ($r = 0.767$, $P = 0.0036$), implicating a role of this subpopulation in Th17 differentiation *in vivo*.
Conclusions: In summary these findings indicate an important role of the $CD14^{++}/CD16^{+}$ monocyte subpopulation in generating a pro-inflammatory milieu to promote the differentiation of Th17 cells in RA patients.

P0294

The hypoxic environment induces a migratory or a proinflammatory phenotype in DCs depending on their maturation stage. Identification of TREM-1 as a common hypoxia marker

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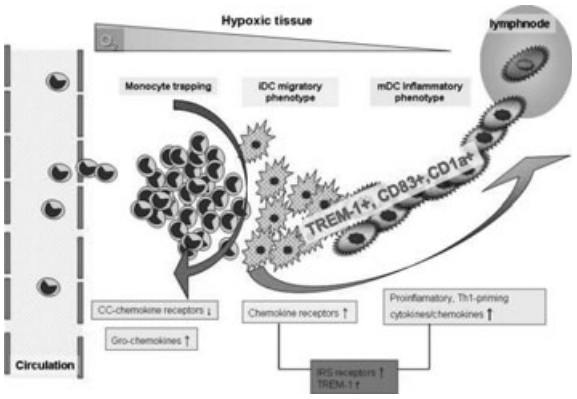
Purpose/Objective: DCs are central to the orchestration of innate/adaptive immunity and maintainance of self-tolerance. DC functions are acquired during a complex differentiation and maturation process tightly regulated by signals present in the microenvironment, and deregulated DC responses may result in amplification of inflammation, loss of tolerance, or establishment of immune escape mechanisms. Pathological conditions are characterized by low partial oxygen pressure (pO_2, hypoxia). Understanding DC biology in low O_2 environment may open new therapeutic opportunities for inflammation and cancer.
Materials and methods: We defined the hypoxic transcriptome of immature (i) and mature (m) DCs generated from human monocytes under chronic hypoxic conditions (5 days, 1% O_2).
Results: We present data showing that chronic hypoxia differentially reprograms DCs depending on their maturation stage. Specifically, hypoxia promotes the onset of a migratory phenotype in iDCs through the upregulation of chemokine receptors and an inflammatory state in mDCs by increasing production of proinflammatory, Th1-priming chemokines/cytokines. Interestingly, hypoxia induces profound changes in the expression of a significant cluster of genes coding for immunoregulatory receptors in both cell subsets. Among them, we identified the triggering receptor expressed on myeloid cells (TREM)-1, a member of the Ig receptor family and a strong amplifier of inflammation, as a hypoxia-inducible gene in both iDCs and mDCs *in vitro* and confirmed TREM-1 expression *in vivo* in DCs infiltrating the

inflamed hypoxic joints of Juvenile Idiopathic Arthritis patients. TREM-1 inducibility by hypoxia was reversed by cell reoxygenation and mediated by HIF-1a. TREM-1 engagement elicited DAP12-linked signaling resulting in the production of proinflammatory cytokines/chemokines and upregulation of T cell costimulatory molecules and chemokine homing receptors.



Conclusions: These findings indicate that reduced O_2 availability critically contributes to the persistence and amplification of inflammation by regulating iDC migration and mDC capacity to promote leukocyte trafficking in diseased tissues and identify TREM-1 as a novel marker of hypoxic DCs endowed with pro-inflammatory properties. The potential implications of DC reprogramming by hypoxia for disease progression will be discussed.

P0295

The LILRB1 inhibitory receptor specific for HLA class I molecules regulates macrophage activation in response to tumour cells

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Purpose/Objective: The loss of HLA class I (HLA-I) expression by tumour cells impairs T cell-mediated recognition but promotes the response of NK cells, which are controlled by inhibitory receptors specific for HLA-I. We addressed whether macrophage (MØ) inhibitory receptors specific for HLA-I (LILRB1 and LILRB2) regulate as well their interaction with tumour cells.

Materials and methods: Monocyte-derived M1 and M2 MØ, differentiated respectively with GM-CSF and M-CSF, were co-cultured with HLA class I (HLA-I) deficient tumour cell lines (721.221, K562) transfected with different HLA-I molecules. MØ activation was monitored analysing cytokine secretion (IL-6, IL-8, TNFa and IL-10) and surface expression of co-stimulatory molecules (CD80, CD86).

Results: Both M1 and M2 MØ expressed surface LILRB1, whereas LILRB2 expression was only detected in M2. Interaction with HLA-I-deficient cell lines triggered cytokine secretion and enhanced CD80 surface expression in both MØ cell types. The response was inhibited upon co-culture with target cells transfected with HLA-G or HLA-B27, but not with HLA-Cw15 or *Cw35alleles, thus suggesting that HLA-I molecules interacting with LILRB1 repressed macrophage activation. Altering target cell HLA-I conformation by acid pH treatment, which prevented LILRB1 binding, restored M2 MØ cytokine secretion. Similar results were obtained by antagonizing the interaction with HLA-I with soluble LILRB1-Fc, but not with LILRB2-Fc.

Conclusions: These data support that LILRB1 plays a dominant role in the HLA-I dependent regulation of MØ activation, and that an altered expression of HLA-I in tumours may promote cytokine production by infiltrating MØ.

P0296

The PI3K/PTEN pathway governs alternative activation and Arginase I expression in macrophages

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Purpose/Objective: Innate immune responses are tightly regulated in order to quickly respond accordingly to invading pathogens. Signaling pathways such as the IKK/NFkB pathway and the MAP kinase pathways are activated in response to pathogens or tissue damage resulting in the expression of a plethora of cytokines and chemokines. We could previously show that the macrophage specific PTEN deficiency limits the excess production of pro-inflammatory mediators and protects from host tissue damage. The aim of our study was to investigate potential mechanisms contributing to the anti-inflammatory phenotype observed in PTEN deficient macrophages.

Materials and methods: In addition to the pre-existing $PTEN^{fl/fl}$ LysM cre mice, we newly generated $PTEN^{fl/fl}$ Arginase $I^{fl/fl}$ LysM cre mice. Furthermore we had access to a potent Arginase inhibitor, N-hydroxy-nor-L-Arginine, and recombinant human pegylated Arginase I. Macrophages were either isolated as thioglycollate elicited macrophages or differentiated to macrophages from bone marrow with M-CSF or conditioned L929 medium. Pure LPS or CpG DNA (ODN 1668) were used to activate macrophages *in vitro*.

Results: In the present study we could provide evidence for differential gene expression in response to TLR agonists suggesting that the PI3K and PTEN are important molecules for M1/M2 macrophage polarization. One of the most potently upregulated genes in PTEN deficient macrophages we identfied was the urea cycle enzyme Arginase I. Arginase I is a signature molecule for alternatively activated macrophages and exhibits important immunological properties.Pharmacologic inhibition as well as cell type specific ablation of the Arginase I gene in macrophages abrogated the protective phenotype observed in PTEN deficient cells. Furthermore we found that vast amounts of Arginase I were secreted by PTEN gene ablated macrophages. Extracellular Arginase I via secondary metabolites could influence not only macrophage activation but also T-cell functions. Using recombinant Arginase I our data suggest that secreted Arginase I confers immune-modulatory effects on macrophages by the reduction of pro-inflammatory cytokines.

Conclusions: Taken together we have ample evidence that PI3K/PTEN govern macrophage polarization and enhanced Arginase I expression contributes to the down-regulation of pro-inflammatory cytokines in PTEN deficient macrophages.

P0297

The role of folate receptor ß-positive macrophages in diseased tissue

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Purpose/Objective: Macrophages display remarkable plasticity that allows them to efficiently respond to environmental signals and change

their phenotype. In chronic inflammatory diseases, such as rheumatoid arthritis (RA), upregulation of several proinflammatory cytokines is responsible for classical (M1) macrophage activation that significantly contribute to the pathology of the disease. Folate receptor β (FRβ) was described as a marker of activated macrophages in RA-affected synovia. However, FRβ expression was also detected in tumour-associated M2 macrophages that show immunoregulatory and tissue repair phenotype.

Materials and methods: To learn which macrophage subset expresses FRβ and to clarify the function of these cells, we differentiated macrophages from human peripheral blood monocytes. We determined their phenotypic characteristics and phagocytic capacity by flow cytometry, measured released cytokines and tested their stimulatory capability by coculture with T cells.

Results: Macrophages differentiated with GM-CSF failed to express FRβ. On the other hand, differentiation using M-CSF produced macrophages highly positive for FRβ. The subsequent activation of the latter by various stimuli showed minor effect on FRβ expression, but revealed existence of functionally different subtypes.

Conclusions: By gaining the possibility to generate *in vitro* macrophages that resemble those in diseased tissues we can comprehensively study their biology and find ways for their elimination or reprogramming.

P0298

The role of protein kinase D enzymes in the innate immune system

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Purpose/Objective: Protein kinase D (PKD) is a novel family of serine/threonine kinases that belong to the calcium/calmodulin-dependent kinase superfamily. Recent research has implicated these enzymes in a vast array of important cellular signaling pathways and downstream functions including cell proliferation, survival, motility and more. Interestingly, a recent role for PKD enzymes has been established in the adaptive immune system whereby murine PKD2 was shown to be essential for normal peripheral T cell function *in vitro* and normal humoral immune responses *in vivo*.PKD enzymes also have a role in regulating innate immune responses in murine/human myeloid lineage cells in Toll-like receptor signaling.

Although the activation and regulation of PKD isoforms is well characterized, little is known about the true physiological roles of these enzymes with regard to myeloid cell development and function.

Materials and methods: Using novel and specific PKD inhibitors in combination with genetically altered PKD mutant mice we are interested in the role of PKD isoforms in macrophage and dendritic cell development, activation responses and effector functions.

Results: Our preliminary data suggests that both PKD1 and PKD2 isoforms are involved the development and effector functions of macrophage and dendritic cells. Using flow cytometry to assess specific myeloid cell populations in the hematopoietic compartment of mice expressing catalytically inactive PKD1 and/or PKD2 alleles we see increased frequencies of conventional DCs and decreased frequencies of macrophages and neutrophils in the spleens of these mutant mice. We also observed that bone marrow-derived dendritic cells produced from PKD2-catalytically inactive mice express higher levels of co-stimulatory molecules such as CD80 and CD86 in response to TLR4 stimulation.Interleukin-6 production by TLR4-stimulated PKD2-catalytically inactive bone marrow-derived macrophages and dendritic cells is also considerably higher than that produced by wild-type cells.

Conclusions: Overall these results suggest that mammalian PKD enzymes may be involved in regulating the innate immune response(s) of myeloid cells.

P0300

The role of vascular-adhesion-protein 1 (VAP-1) in mediating monocyte migration across inflamed hepatic sinusoidal endothelium

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Purpose/Objective: Monocyte progeny cells drive liver fibrosis. The $CD14^{+}$ $CD16^{++}$ monocyte subset is believed to be pro-inflammatory whereas $CD14^{++}CD16^{-}$ monocytes maybe reparative. The molecular basis of monocyte transmigration across hepatic sinusoidal endothelial cells (HSEC) into the liver is poorly understood. VAP-1 is an atypical adhesion molecule and an ectoenzyme with monoamine oxidase activity that is involved in the recruitment of hepatic lymphocytes. We now report that VAP-1 and its ectoenzyme activity differentially regulate the recruitment of pro-inflammatory monocytes across HSEC.

Materials and methods: Human HSEC were isolated from explanted livers. After TNF-α/IFN-γ stimulation HSEC were treated with VAP-1 antibody or enzyme inhibitors. Peripheral monocytes were enriched using density gradient. Monocyte subsets were obtained via FACS-sorting. Monocytic transendothelial migration was analyzed in a flow assay. Transwell assays were used to study phenotypic changes in transmigrated monocytes. Surface marker expression was studied by FACS and imaging the route of monocyte transmigration across HSEC (transcellular versus paracellular) was performed by confocal microscopy.

Results: Inflamed HSEC displayed enhanced adhesion molecule expression (ICAM-1, VCAM-1, VAP-1, CD31). Confocal microscopy revealed an increase of monocytes taking the transcellular route through inflamed HSEC, indicating the translocation of adhesion molecules to transcellular pores. Anti-VAP-1 antibody or enzyme inhibitor both reduced monocyte transmigration under flow but did not alter monocyte phenotype. VCAM-1 blockade had a similar but redundant impact and PTx and ICAM-1-inhibtion further impacted transmigration. Blocking VAP-1 led to a profound reduction in $CD14^{+}$ $CD16^{++}$ transmigration but also affected $CD14^{++}CD16^{+}$ cells whereas $CD14^{++}$CD16- cells were not affected. Monocyte subsets differentially expressed the potential VAP-1 ligands SIGLEC-9/-10. Under static conditions the effect of VAP-1 was significantly blunted suggesting that flow is a prerequisite for its biological function.

Conclusions: Endothelial VAP-1 differentially modulates monocyte recruitment under flow in a time-dependent fashion favouring transmigration of a proinflammatory monocyte subset, but other adhesion molecules and chemokine-mediated signals significantly contribute to monocyte transmigration across inflamed HSEC. The critical role of VAP-1 ectoenzyme function implies that small molecule enzyme inhibitors maybe effective in combating liver inflammation and subsequent fibrosis.

P0301

The tetraspanin CD37 promotes dendritic cell migration

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Purpose/Objective: Tetraspanins are a family of integral membrane proteins that function by forming tetraspanin-enriched microdomains (TEMs), thus organizing the cell surface. TEM formation allows tetraspanins to function in critical processes including cell adhesion and migration. Immune system aberrations are observed in tetraspanin

deficient mice. T cells and dendritic cells (DC) derived from $CD37^{-/-}$ mice display hyperproliferative and hyperstimulatory phenotypes, respectively. However, interestingly these mice are unable to generate an effective T cell response *in vivo*, following antigenic challenge, including irradiated B16OVA (an ovalbumin expressing tumor cell line). We aim to determine that a defective migratory capability of $CD37^{-/-}$ DCs underlies this poor response.

Materials and methods: The i*n vivo* antigen presenting capability of WT and $CD37^{-/-}$ mice was determined by the adoptive transfer of CFSE-labelled WT OT-I T cells and subsequent challenge of these mice with irradiated B16OVA. T cell proliferation was then determined by flow cytometric analysis. Dermal DC migration following inflammation was visualized by two-photon confocal microscopy of $CD37^{-/-}$CD11c-YFP mice. Finally, integrin expression on WT and $CD37^{-/-}$ BMDC was determined by flow cytometric analysis.

Results: Antigen presenting capability within $CD37^{-/-}$ mice is poor, as demonstrated by the inadequate ability of these mice to efficiently prime WT OT-I T cells *in vivo*. Dermal DC are unable to effectively migrate in a directional manner following induction of inflammation. Finally, expression of a range of integrins is normal on $CD37^{-/-}$ BMDC.

Conclusions: $CD37^{-/-}$ DC poorly prime WT OT-I T cells *in vivo*, consistent with a defect in the migratory capability of these cells. This is supported by the observation that $CD37^{-/-}$ dermal DC do not migrate directionally in an inflamed state. This inability to migrate is not dependant on poor expression levels of integrins. As tetraspanins can regulate integrin outside-in signalling and cytoskeletal rearrangement, we are currently investigating any possible roles for CD37 in these processes.

P0302

Triggering of B7h by the inducible costimulator modulates maturation and migration of monocyte-derived dendritic cells

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Purpose/Objective: B7h, expressed by several cell types, binds Inducible co-stimulator (ICOS) expressed by activated T cells. This work investigated the effect of B7h triggering by ICOS on human monocyte-derived dendritic cells (DC).

Materials and methods: Monocytes were induced to differentiate to immature DC (iDC) by a 5-day culture with GM-CSF and IL-4. They were then induced to mature for 2 days with LPS in the absence (mDC) and the presence (mDC^{ICOS}) of ICOS-Fc, a recombinant form of ICOS fused to the human or mouse IgG1 Fc; the mutant ^{F119S}ICOS-Fc, which doesn't bind B7h, and CTLA-4-Fc were used as controls. Supernatants were then collected for cytokine analysis by ELISA. In the antigen presentation assay, keyhole limpet hemocyanin (KLH) was added to DC with during the maturation step in the presence of the different ICOS-constructs; these DC were then cocultured with autologous T cells, and IFN-gamma production from $CD4^+$ and $CD8^+$ T cells was assessed by ELISpot. In the adhesion assay, endothelial cells (EC) were treated or not with the different ICOS-Fc constructs and incubated with iDC or mDC; adherent cells were then counted by the Image Pro Plus Software. In the migration assay, iDC or mDC were plated onto the Boyden chamber in the presence of appropriate chemoattractants and the different ICOS constructs. Cells on the bottom of the filter were stained with crystal-violet and counted.

Results: Results showed that mDC^{ICOS} produced higher amounts of IL-23 and IL-10, and lower amounts of TNF-α and IL-6 than mDC. Moreover upon pulsing with KLH, mDC^{ICOS} were better stimulators of IFN-gamma production in $CD8^+$ T cells than mDC, whereas no difference was found in $CD4^+$ T cells. Finally, ICOS-Fc inhibited adhesion of both iDC and mDC to EC, and their migratory activity.

Conclusions: These data suggests that 'reverse' signaling mediated by B7h modulates DC maturation by influencing cytokine secretion and potentiating antigen cross-presentation. Moreover, it modulates DC adhesiveness and migration and might thus influence their recruitment into tissues.

P0303

Wnt5a plays an essential role during human monocyte-derived dendritic cell maturation and function

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Purpose/Objective: Recent evidences reveal an immunomodulatory role for Wnt5a. *In vivo* Wnt5a levels increase under numerous pro-inflammatory situations; being detected in human inflammatory diseases and raise in the sera from patients with sepsis. Our previous results demonstrate that Wnt5a interfere in dendritic cell (DC) differentiation from human monocytes (MoDCs) leading to unconventional DCs with tolerogenic features. In the present work we analyse the role of Wnt5a during DC maturation and function.

Materials and methods: Immature DCs were obtained from human $CD14^+$ monocytes and stimulated by LPS treatment. Changes during DC maturation in the pattern of expression of non canonical Wnts and their receptors were determined by PCRq. The effect in DC phenotype and function of an increase in Wnt5a signaling, previous or simultaneous to LPS response was analysed. The production of Wnt5a by DC was silenced with specific pre-designed siRNAs to analyze the impact of Wnt5a drop during DC maturation and function.

Results: Wnt5a is the main non canonical Wnt protein expressed by MoDCs and LPS signaling stimulated its production. Nevertheless, Wnt5a alone was not able to induce DC phenotypic maturation or cytokine secretion but improved their endocytic capability. Moreover, in response to LPS pre-treated Wnt5a-DCs secreted higher levels of IL-12 but lower of IL-10 and MLR assays showed lower production of IL-10 by naïve T cells primed with pre-treated Wnt5a-DCs. The addition of Wnt5a during DC LPS-maturation increased also IL-12 secretion and reduced that of IL-10 but its impact on DC allostimulatory capabilities was irrelevant suggesting a main role for autocrine Wnt5a. Accordingly, Wnt5a-silenced DCs showed a reduced viability and failed to produce pro-Th1 cytokines while the secretion of IL-10 was increased. Consequently, these Wnt5a-silenced DCs showed a reduced capacity to induce Th1 responses promoting IL-10 secretion by CD4 T cells. This situation was rescued by the addition of Wnt5a.

Conclusions: Although our previous results demonstrate that the increment Wnt5a signaling on monocytes interfere with their differentiation into DCs, the data presented herein indicate a positive and necessary function for Wnt5a on immature and mature DCs suggesting a timing-dependent dual role for Wnt5a on human monocyte derived DCs.

P0304

Zinc oxide nanoparticles and monocytes: impact of size, charge and solubility on activation status

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Purpose/Objective: The abundance of products containing ZnO nanoparticles (ZnO NPs) available to the consumer poses an important question of safety. Therefore our objectives were firstly to assess ZnO NP induced cytotoxicity with respect to size, charge and solubility on the monocytic cell line THP-1. Secondly, to determine how these factors at sub lethal concentrations influence the activation of these cells.

Materials and methods: We have used three manufactured ZnO NP dispersions (average diameter 70 nm: anionic, cationic and non ionic) and compared their effects on THP-1 cells with bulk ZnO (<44 μm mesh) and equimolar $ZnSO_4$. Three different cytotoxicity/viability assays were evaluated: release of lactate dehydrogenase (LDH), WST-1 conversion to formazan and nuclear binding of 7-Amino-actinomycin D (7-AAD). THP-1 cells were exposed for 24 h to a dose range of 6.25−300 $\mu g/10^6$ cells. Dissolution of ZnO NPs was assessed in the presence or absence of 10% foetal bovine serum (FBS) using flame atomic absorption spectroscopy (AAS) and centrifugal filtration. The immunomodulatory effect of the NPs was assessed by cytokine ELISA and surface marker expression by flow cytometry.

Results: ZnO NPs are more toxic than the bulk form and a positive charge enhances cytotoxicity of the NP despite their relatively high dissolution. Centrifugal filtration (cut off of 5kDa) and Zn element analysis by AAS confirmed that in the absence of cells $ZnSO_4$, bulk and NP ZnO to 10% FBS resulted in a strong association of the Zn^{2+} ions with protein. Following 24h exposure of THP-1 cells at sublethal concentrations of these Zn particles there was no effect on immunological markers of inflammation such as HLA-DR and CD14. There was a modest increase in the adhesion molecule CD11b. There was no induction of the classical pro-inflammatory cytokine TNFα and also no effect on LPS-stimulated TNFα production.

Conclusions: These results suggest that ZnO NP despite their toxicity do not induce a classical pro-inflammatory effect on THP-1 cells.

Poster Session: Sensing – NLRs, IL1 & Inflammasomes

P0305
Activation of inflammatory pathways by apoptotic inducers

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Purpose/Objective: Inflammation and cell death play key roles in numerous conditions including stroke and Alzheimer's disease. Although both processes act through caspases, they are conventionally considered to be distinct pathways. Recent studies, however, have highlighted interactions between the two, such as the regulation of NLRP3 activation by oxidised mitochondrial DNA and by the anti-apoptotic protein Bcl-2, plus the ability of the AIM2/ASC complex to initiate a caspase-8-dependant apoptosis in the absence of caspase-1. The purpose of this work was to investigate interactions between the apoptotic and IL-1β processing pathways by investigating cell death and IL-1β release induced by DAMPs and apoptosis inducers.
Materials and methods: Bone marrow-derived macrophage cell lines (WT and NLRP3 KO) were treated with either DAMPs or one of a range of apoptosis inducers for 1 to 24 h, with or without a 4 h LPS pre-treatment. Caspase involvement was determined with the use of either specific or broad-spectrum inhibitors. Cell death was measured using the LDH assay, and IL-1β release quantified by ELISA. IL-1β was characterised as pro- or mature by Western blot.
Results: Treatment with DAMPs such as ATP, sphingosine or monosodium urate crystals resulted in cell death that was not affected by LPS priming. Mature IL-1β was released from LPS-primed cells treated not only with known DAMPs such as ATP or sphingosine, but also with the apoptotic inducers staurosporine or TPEN. A significantly lower level of IL-1β release was seen for other apoptosis inducers, which caused significant levels of cell death.
Conclusions: Induction of apoptosis can result in the processing of IL-1β, i.e. initiation of inflammatory pathways. As this effect was not equal across apoptosis inducers, it cannot be considered as a general apoptotic event, but is rather related to a specific action of some of these toxins. These differences could be used to identify new mechanisms driving inflammation.

P0306
Activation of the inflammasome by fungal immunomodulatory proteins LZ-8 and GMI

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Purpose/Objective: IL-1β is a key proinflammatory cytokine regulating both innate and adaptive immune responses, and its production is controlled by the inflammasome. Inflammasomes are multiprotein platforms responsible for caspase-1 activation and subsequent processing and secretion of mature IL-1β. Here we studied the stimulatory effect of fungal immunomodulatory proteins (FIPs) LZ-8 and GMI on inflammasome activation and IL-1β production in murine macrophages.
Materials and methods: Murine peritoneal resident macrophages or bone marrow-derived macrophages were treated with recombinant LZ-8 and GMI proteins in the presence or absence of LPS, and the production of caspase-1 and mature IL-1β were evaluated. C57BL/6 and IL-1R$^{-/-}$ mice were treated with LZ-8 before *Listeria monocytogenes* challenge to assess the protective function of LZ-8 against bacterial infection in animals.
Results: FIP stimulation resulted in caspase-1 activation and robust IL-1β production in LPS-primed macrophages. FIP-induced IL-1β production was inhibited in the presence of high extracellular KCl or inhibitors targeting the membrane pores P2X7 and pannexin-1, indicating that FIP-induced inflammasome activation requires potassium efflux through ion channels. In addition, inhibition of reactive oxygen species (ROS) production also reduced FIP-induced IL-1β production, indicating that ROS generation is also involved in the process. Intraperitoneal administration of LZ-8 in mice elicited a robust influx of neutrophils which was attenuated in IL-1R$^{-/-}$ mice, and this immune response could protect mice against subsequent *L. monocytogenes* infection.
Conclusions: Our findings provide evidence that FIPs can activate the inflammasome formation and IL-1β secretion, leading to a protective immune response against bacterial infection.

P0307
Analysis of inflammasome related molecules in the response to H5N1 Avian Influenza

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Purpose/Objective: Highly pathogenic avian influenza (HPAI) infection is extremely acute and associated with severe mortality in both humans and poultry. The rapid onset of disease suggests that virus-host interactions, such as the immune response to virus, might contribute to the severity. With this in mind, it is critical to understand the host-pathogen interactions in order to develop novel approaches to managing infection. Pro-inflammatory cytokines, such as interleukin (IL)-1β and IL18, are crucial to an anti-viral response, however, the control of the expression of these molecules is vital, as exacerbated levels can lead to deleterious outcomes. In mammals these cytokines are regulated by a complex cytoplasmic protein scaffold known as the Nalp3 inflammasome. Currently, the mechanisms for Nalp3 activation are largely unknown, however, it appears Nalp3 and the associated cleavage enzyme caspase 1 (ICE) are important regulators. To elucidate the role of the inflammasome in the chicken response to H5N1 HPAI, we identified and characterized chicken Nalp3, ICE, IL1β and IL18 and investigated their role during infection.
Materials and methods: Through qRT-PCR, siRNA gene knockdown and ELISA analysis, the chicken Nalp3 inflammasome has been identified and its role in the response to H5N1 HPAI analysed.
Results: ChickenIL-1β, IL-18, Nalp3 and ICE are upregulated during H5N1 infection, particularly Nalp3 and ICE. In correlation with this was an observed increased in IL-1β, IL-18 and IFN-γ protein secretion, measured in chicken sera.
Conclusions: Together, these data indicate a strong Nalp3 inflammasome response; following HPAI infection in the chicken.This work provides an insight into strategies that target the immune system for improving resistance to avian influenza.

P0308
Apis mellifera (honey bee) venom activates the AIM2 inflammasome in primary keratinocytes – a first step to allergic sensitisation?

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Purpose/Objective: Following allergen exposure, cytokines and other pro-inflammatory signals play an important role in the immunological cascade leading to allergic sensitisation. Inflammasomes sense exoge-

nous and endogenous danger signals and trigger IL-1β and IL-18 activation which in turn shape Th2 responses. Honey bee venom (BV) allergies are very common however, the local inflammatory cascade leading to initiation of allergic sensitisation towards BV is poorly understood. In this study the local inflammatory cascades in the skin after exposure to BV were investigated.

Materials and methods: In human skin and in cultured primary keratinocytes the mechanisms of inflammasome activation upon BV exposure were analysed by ELISA, Western Blot, flow cytometry, siRNA techniques and immunofluorescence.

Results: In an *ex vivo* bee sting model BV induced IL-1β release suggesting activation of an inflammasome. Indeed, in cultured primary keratinocytes the BV component melittin triggered IL-1β and IL-18 release via the AIM2 inflammasome whereas the NLRP3 inflammasome remained unaffected. AIM2 is a cytosolic DNA receptor and mitochondrial as well as genomic DNA was detected in the cytosol of melittin treated keratinocytes as triggers of inflammasome activation. As a mechanism, melittin specifically mediated destruction of mitochondrial membranes leading to leakage of mitochondrial DNA into the cytosolic compartment which subsequently activated AIM2.

Conclusions: These data suggest that upon BV exposure keratinocytes are involved in a primary immune response by activation of the AIM2 inflammasome triggered by endogenous DNA resulting in a subsequent IL-1β and IL-18 release. As IL-1β and IL-18 are critically involved in Th2 and IgE mediated immune reactions these results could add to the understanding of the role of the local tissue microenvironment to subsequent allergic responses in predisposed individuals.

P0309
ATP-induced inflammasome activation in LPS-primed bovine monocytes is independent of the purinergic receptor P2X7

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Purpose/Objective: Inflammasome-mediated release of IL-1§ from ATP-stimulated human and murine monocytes requires the expression of the the ATP-gated ion channel subtype P2X7 receptor. We investigated the role of the purinergic receptor P2X7 for inflammasome activation in bovine monocytes.

Materials and methods: ATP-induced inflammasome assembly in bovine monocytes was analyzed by flow cytometric evaluation of caspase-1 activation and the measurement of IL-1b release with ELISA after LPS^+ATP-stimulation. To evaluate the function of the cell surface pore-forming ATP receptors in bovine MNC, agonist-induced ethidium bromide (EB) uptake in MNC was assessed using time-resolved flow cytometry. To investigate the role of Ca^{2+} influx for ATP-induced inflammasome activation, bovine MNC were Fluo-4-loaded and a continuous measurement of cytosolic calcium was measured by flow cytometry. Cells were loaded with the dye DHR-123 to analyze the generation of reactive oxygen species (ROS) by flow cytometry.

Results: The release of IL-1b was minimal when MNC were stimulated with LPS alone but sixfold higher in the presence of ATP. Inhibition of caspase-1 activation with a pan-caspase-inhibitor completely blocked IL-1b release. ATP, BzATP and 2-MeSATP induced pore formation and uptake of EB in bovine lymphocytes but not in monocytes or B cells. A human P2X7 receptor antagonist inhibited the EB uptake of ATP stimulated $CD4^+$ T cells but not the IL-1b release from bovine MNC. All ATP isomers induced ROS generation in bovine lymphocytes with BzATP being the most effective one. In monocytes only PMA but none of the three ATP isomers was able to induce ROS. In addition, ATP and 2-MeSATP but not the high affinity P2X7 agonist BzATP induced calcium influx in bovine monocytes. Inhibition of K^+ efflux by high extracellular K^+ concentrations completely blocked the LPS^+ATP-induced release of IL-1b from bovine MNC.

Conclusions: P2X7 mediated pore formation was observed in subsets of bovine T lymphocytes ($CD4^+ > CD8^+$) but not in monocytes or in B cells. The data indicate that neither ROS generation nor Ca^{2+} influx play a role in the ATP-induced release of IL-1b from bovine monocytes, which is independent of the purinergic receptor P2X7.

P0310
Deubiquitinating enzymes: new players in IL-1beta release

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Purpose/Objective: Ubiquitination is a post-tranlational modification essential for maintaining cellular homeostasis. The classical role of ubiquitin is to target proteins for degradation by the proteasome. Ubiquitin molecules are recycled by the cell prior to protein degradation by deubiquitinating enzymes (DUB).DUBs present specificity towards different ubiquitin chain topologies and are important regulators of key cellular processes such as cellular trafficking, gene transcription and cellular signalling.

IL-1beta is a potent proinflammatory cytokine that is synthesised as a precursor molecule upon pathogen or danger associated molecular pattern (PAMP and DAMP) stimulation. Once synthesised this cytokine has to be processed by caspase-1 in order to become active and secreted. This activation occurs in the cytosol within a molecular complex called the inflammasome. The NLRP3 inflammasome is the best studied so far and its assembly can be triggered by a wide variety of stimuli.However the signalling pathways by which these stimuli lead to the assembly of the inflammasome complex are not completely understood.

The aim of this study was to investigate the role of DUBs in inflammasome activation.

Materials and methods: For this study we pre-treated murine peritoneal macrophages and the human cell line THP-1 with different DUB inhibitors and study the effect of this compounds in the ATP and nigericine induced processing and release of IL-1beta by western blot and ELISA.

Results: Here we report that DUBs play a crucial role in inflammasome activation. We have found that inhibition of DUBs with the inhibitors eeyarestatin I, b-AP15 and WP1130 block the processing and release of IL-1beta in macrophagesinduced by the NLRP3 inflammasome activators ATP, nigericin and monosodium urate crystals (MSU). These inhibitors do not block activity of recombinant caspase-1 *per se* but impair the formation of the ASC pyroptosome suggesting that DUBs act upstream caspase-1 activation. This is a proteasome independent process since inhibition of the proteasome with the clinically used Bortezomib does not impair this process.

Conclusions: All these data suggests that deubiquitination plays an essential role in inflammasome assembly and IL-1beta release indicating a new function for DUB activity in the innate immune response.

P0311
Ethanol inhibits NLRP3 and AIM2 inflammasome activation

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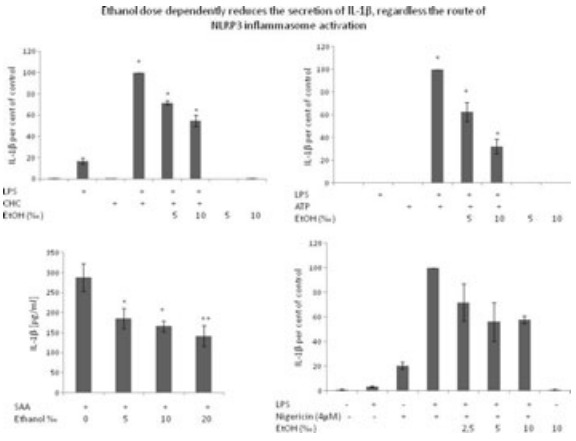
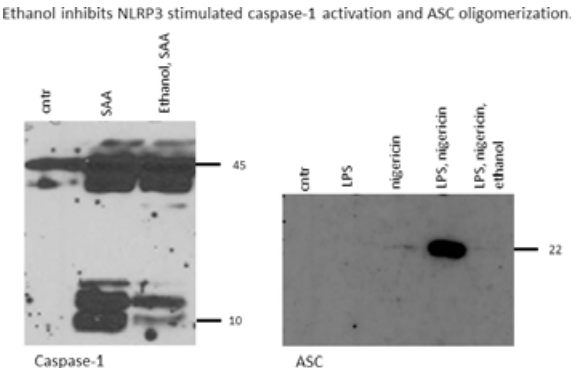
Purpose/Objective: Local macrophage driven inflammation and the secretion of proinflammatory cytokines is recognized as a major driving force in the pathogenesis of atherosclerosis. Acute ethanol exposure has been shown to reduce the release of proinflammatory cytokines. We examined whether ethanol would modulate the activa-

tion of NLRP3 and AIM2 inflammasomes and subsequent secretion of IL-1β in cultured human macrophages.

Materials and methods: Primary human macrophages and THP-1 cell line cells were used. NLRP3 inflammasome was activated by cholesterol crystals, ATP, Serum amyloid A (SAA) and nigericin. AIM2 inflammasome was activated with Poly (dA:dT). The secretion of IL-1β was measured by ELISA and Western blot assays. Activation of caspase-1 and ASC was studied by Western blotting. The lysosomal rupture and release of cathepsin B was imaged by fluorescent staining. Cholesterol crystal esterification was judged by TLC. mRNA expressions were analyzed by quantitative real time PCR.

Results: Ethanol decreased dose-dependently the production of mature IL-1β induced by the activators of NLRP3 and AIM2 inflammasomes. Ethanol reduced the NLRP3 inflammasome activation induced by chemically diverse inducers, ATP, cholesterol crystals, serum amyloid A and nigericin. Decreased secretion of IL-1β paralleled the reduction of the activation of caspase-1, an enzyme which cleaves the proIL-1β into the mature form. Ethanol also attenuated the secretion induced by synthetic double stranded DNA, an activator of AIM2 inflammasome. Ethanol attenuated the disruption of lysosomal integrity and the leakage of lysosomal protease cathepsin B. Ethanol also reduced ASC oligomerization. Ethanol had no significant effect on the expression of NLRP3 or proIL-1β mRNA in LPS-primed macrophages, suggesting that ethanol inhibits the inflammasome activation instead of synthesis of proIL-1β. In contrast, acetaldehyde, a highly reactive metabolite of ethanol, had no effect on the ATP-induced IL-1β secretion.





Conclusions: Provided that ethanol also inhibits macrophage inflammasome activation *in vivo* the present findings provide a plausible mechanistic explanation for the association between moderate alcohol consumption and reduced incidence of cardiovascular diseases.

P0312

Identification of novel NOD2-regulated proteins using phosphoproteomics

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Purpose/Objective: NOD2 is an intracellular pattern recognition receptor (PRR) that recognizes muramyl dipeptide (MDP). Variants in the NOD2 ligand recognition domain have been found to be associated with a significant percentage of Western Crohn's disease, but the mechanisms leading to disease are so far unknown. Our objective was to define the molecular mechanism of NOD2 signalling by performing a phosphoproteomic analysis of the NOD2 signalling cascade. Cell activation was enhanced by stimulating cells with both MDP and TLR2 ligand PAM3cys, which strongly synergizes with MDP.

Materials and methods: Human monocyte-derived dendritic cells from five different donors were left untreated or stimulated with MDP, PAM3cys or both. Eluates were subjected to tryptic digest before mass spectrometry analysis by LC-MS/MS and Q-TOF. Identified phosphoproteins that showed a > 1.5-fold increase or <0.67-fold decrease relative to control sample were considered significantly regulated. The regulation of the individual proteins was validated by examining phospho-enriched samples on Western blot.

Results: Different treatments resulted in distinct patterns of regulated proteins. As expected, stimulation with both MDP and PAM3cys resulted in an increased number of regulated proteins compared to MDP alone. We found several significantly regulated proteins previously described to be involved in NOD2 signalling. Identified regulated proteins were, among others, associated with NFκB activation, autophagy induction, endosomal transport, proteasomal degradation and MHC presentation. The MDP-induced regulation of l-plastin, heat shock protein beta1, zyxin and NCF1 has been confirmed by Western blot.

Conclusions: The phosphoproteomic analysis of the NOD2 signalling cascade is validated by the retrieval of proteins previously described in NOD2 signalling. Identified regulated proteins are associated with a wide range of immunological functions. We have thus far been able to identify and confirm l-plastin, heat shock protein beta1, zyxin and NCF1 as proteins involved in NOD2 signalling. Currently the functional role of each protein identified is being assessed in terms of bacterial handling, antigen presentation and cytokine production.

P0314

Intestinal manipulation during surgery causes sterile inflammation and post-operative ileus via mast cell protease activity — dependent IL-1receptor signalling

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Purpose/Objective: Intestinal manipulation (IM) of the bowel surgery commonly causes a transient post-surgical cessation of gut motility, i.e. postoperative ileus (POI). We have previously shown that POI is mediated by activation of mast cells. We studied the role of mast cells and IL1signaling routes as causal factors for POI.

Materials and methods: WT C57Bl/6 mice, TLR$^{-/-}$, MyD88 $^{-/-}$, and mast cell deficient Kit/wsh mutants were used. In a mouse model for POI, mice underwent laparotomy (L) or manipulation of the small bowel (IM). After surgery, intestinal transit and inflammation was assessed.

Results: Surgical manipulation (IM) led to a neutrophil infiltrate in the intestinal muscle layer in WT mice (L: 21 ± 10; IM: 219 ± 24 cells/mm^2), associated with a significantly enhanced production of cytokines IL1 alpha, IL1 beta, CCL-2, IL-6 and KC in the muscle layer. This inflammatory infiltrate after IM was significantly attenuated in mast cell deficient Kit/wsh mice.

Muscular inflammation in POI was dependent on MyD88 signaling, as neutrophil recruitment (IM-WT: 219 ± 24 versus IM-MyD88$^{-/-}$:80 ± 18 cells/mm^2) as well as production of CCL2, and IL-6 was significantly reduced in MyD88$^{-/-}$ undergoing IM. MyD88 is crucial in TLR and IL-1R signaling. However, TLR 2, 4, 2/4, and 9 $^{-/-}$ mice displayed no change in IM-induced neutrophil infiltrate after IM. In contrast, treatment with the IL-1R antagonist (Anakinra) significantly reduced muscularis IL-6 and CCL2 production and neutrophil recruitment, and ameliorated intestinal transit. Interestingly, IM-induced POI was unaffected in mice deficient in inflammasome proteins NALP3 or ASC1, indicating that Il1beta is cleaved by inflammasome independent pathways. Further, we show that active IL-1beta production was significantly inhibited in mast cell deficient mice intestinal muscularis. *In vitro*, conditioned medium from a peritoneal mast cell culture elicited Il1beta release in bone marrow dendritic cells. In conjunction, treatment of mice with mast cell proteinase inhibitor chysmostatin inhibited colonic IM-induced CCL2 and IL-6 production significantly.

Conclusions: IM during abdominal surgery induces mast cell activation, and an inflammatory infiltrate via an IL1R dependent pathway. Our data further suggest that POI arises via mast cell proteinase activity that cleaves and activates IL-1 in the manipulated small intestinal muscularis.

P0315
Lactate induces caspase-1-independent release of interleukin-1 beta

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Purpose/Objective: Lactic acidosis is a clinical consequence and contributor to many major diseases. Inflammation, and in particular the pro-inflammatory cytokine interleukin-1 beta (IL-1b), contributes to the development and exacerbation of multiple diseases; including epilepsy, Alzheimer's disease and stroke. IL-1b is synthesised as an inactive precursor which is subsequently cleaved to a 17 kDa active form by caspase-1. To cleave IL-1b, caspase-1 also requires prior activation. This occurs through association with a large protein complex termed the 'inflammasome'. To determine whether acidosis affects IL-1b processing, we examined the effect of lactate on IL-1b release from glia.

Materials and methods: Mixed glial cultures were grown from wild type and NLRP3 knock-out mice. Pro- and mature IL-1b release was measured following addition of 25–55 mM lactate with, or without, pre-treatment with the caspase-1 inhibitor, YVAD-CHO (Calbiochem).

Results: 25 mM lactate induced the release of IL-1b from mouse glial cultures. IL-1b release was not significantly altered by pre-treatment with YVAD-CHO. Lactate-mediated IL-1b release also occurred in NLRP3 knock-out cultures. The lactate-induced IL-1b release is distinct from the caspase-1 dependent process since the mature IL-1b present in treated supernatants is 20 kDa as opposed to 17 kDa.

Conclusions: Lactate induced IL-1b release from glial cells and this release was independent of the classical NLRP3 inflammasome/caspase-1 pathway. Further investigation of this caspase-1-independent IL-1b pathway may in future provide novel targets for the treatment of inflammatory disease.

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P0317
NLRC$_4$ mediates protection in bacterially triggered intestinal inflammation

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Purpose/Objective: Pattern recognition receptors (PRRs) enable mammals to discriminate self from non-self through the recognition of microbial signatures, such as bacterial flagellin. Flagellin is detected by the PRR NLRC4, which drives the activation and release of potent pro-inflammatory mediators. We aimed to characterize the role of NLRC4 in bacterially-triggered intestinal inflammation.

Materials and methods: We used a model based on the intestinal pathogen *Citrobacter rodentium*, a close relative of enteropathogenic *E. coli,* that induces acute intestinal pathology and diarrhea in mice.

Results: Following oral infection with *C. rodentium*, NLRC4$^{-/-}$ mice developed more severe weight loss, increased bacterial colonization levels and exacerbated intestinal inflammation compared to WT mice. Experiments using bone marrow chimeras revealed that the protective effects of NLRC4 were mediated by non-hematopoietic cells.

Conclusions: In conclusion, NLRC4 signalling in non-hematopoietic cells provided protection from bacterially-induced intestinal inflammation, possibly by limiting bacterial colonization. Further studies are needed to elucidate the molecular mechanisms responsible for protection.

P0318
NLRP3 inflammasome activation by prion protein fibrils

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Purpose/Objective: Transmissible spongiform encephalopathies (prion diseases) are characterized by accumulation of abnormal form of prion protein, spongiform change, gliosis and progressive neuronal cell loss; however, the underlying cause of neurodegeneration is not known. Previous studies have indicated the role of innate immunity, particularly of NLRP3 inflammasome in other amyloid diseases Alzheimer's disease and type II diabetes. Production of proinflammatory cytokine IL-1β has also been observed in brain in several types of prion disease and IL-1R deficiency significantly prolonged the onset of the disease. The aim of our study was to investigate whether prion protein deposits can instigate NLRP3 inflammasome.

Materials and methods: We prepared several types of assemblies of prion protein and biophysically characterized them. Cytokine release from fibril-activated microglia and macrophages from wild-type mice and from mice deficient in NLRP3, ASC and caspase-1 was assessed by ELISA. Confocal microscopy and flow cytometry were used to determine caspase-1 activation, lysosome destabilization and neurotoxicity in co-cultured microglia and neurons.

Results: We show that prion protein fibrils, converted from α- to predominantly β-type conformation, trigger cleavage of pro-IL-1β, which requires the inflammasome components NLRP3, ASC and caspase-1, whereas the native monomeric form of prion protein does not induce activation of IL-1β. Several of the previously proposed NLRP3 inflammasome activation pathways may contribute to prion fibril-induced inflammasome activation, since it can be blocked by inhibition of K^+ efflux and inhibition of phagocytosis. Proteinase K-resistant prion protein fibrils induce release of neurotoxic species by microglial cells and can thus contribute to neuronal cell death.

Conclusions: We demonstrated that prion protein fibrils, but not oligomers or monomers, via activation of NLRP3 contribute to neuronal cell death. Our results and previous studies on IL-1R-deficient mice suggest the IL-1 signaling pathway as the perspective target for the therapy of prion disease.

P0319
NLRP3 inflammasome activity is negatively controlled by miR-223

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Purpose/Objective: Inflammasomes are multiprotein signaling platforms that form upon sensing microbes or endogenous danger associated molecular patterns to activate caspase-1. Activated caspase-1 leads to the secretion of proinflammatory cytokines and the initiation of a special type of cell death, known as pyroptosis. Especially the NLRP3 inflammasome appears to be a general sensor of cell stress. In contrast to other inflammasome proteins, NLRP3 is expressed under limiting conditions in resting cells and an additional priming signal is critically required to boost its expression. This induction of NLRP3 protein expression is licensed by PRR signaling which allows respective NLRP3 activators to trigger caspase-1 cleavage. In general, NLRP3 inflammasome activation is tightly regulated at several steps including the transcriptional control of its expression. Based on the observation that the upregulation of NLRP3 mRNA following a pro-inflammatory signal is rather short-lived we speculated that NLRP3 expression could also be subject to miRNA-dependent posttranscriptional regulation.
Materials and methods: To systematically address this hypothesis, we performed genome-wide screening using a miRNA precursor library to identify microRNAs that regulate NLRP3 expression. These data were correlated with miRNA expression arrays. Screening results were verified in primary cells by loss of function and gain of function experiments.
Results: We identified the myeloid specific miRNA miR-223 as critical regulator of NLRP3 inflammasome activity. miR-223 suppresses NLRP3 expression through a conserved binding site within the 3' UTR of NLRP3. This translates into reduced NLRP3 inflammasome activity.
Conclusions: While miR-223 itself is not regulated by proinflammatory signals, its expression varies among different myeloid cell types. Thereby, given the tight transcriptional control of NLRP3 message itself, miR-223 functions as an important rheostat controlling NLRP3 inflammasome activity.

P0321
Pattern recognition receptor modulation of murine cutaneous wound healing.

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Purpose/Objective: Chronic wounds including pressure sores, venous leg ulcers and diabetic foot ulcers occur in one in twenty of the elderly population greatly affecting quality of life. They cause pain, distress and disability for patients, and pose a continuing challenge to global health care professionals, with estimated annual treatment costs exceeding £3 billion in the UK alone. Emerging data suggest wound colonization with bacterial species including Staphylococcus, Pseudomonas and Enterbacter and ultimately the formation of a biofilm is a key confounding factor in the aetiology of chronic wound ontogenesis and the prolonged pro-inflammatory non-healing phenotype. Despite recent advances in the profiling of microflora in infected wounds, the mechanisms whereby the host responds to (or in cases of pathology fails to respond to) colonising bacteria remain unclear. Over a wide range of epithelia host pattern recognition receptors (PRRs) play a key role in this environmental surveillance through their ability to recognise and respond to a diverse range of pathogen associated molecular patterns (PAMPs). Surprisingly, little is known about host-microflora interaction during skin wound repair. The aim of this study was to explore host modulation of skin microflora in PRR null mice and preclinical delayed healing murine models.
Materials and methods: The bacterial skin flora of normal and wounded skin from each model was profiled by eubacterial 16S PCR-DGGE, in conjunction with DNA sequencing.
Results: We reveal an important interplay between the cutaneous microbiota, the innate immune system and specific skin PRRs. Further, we identify key miroflora changes in delayed healing mouse models.
Conclusions: Our findings support the hypothesis that specific genetic or functional changes in the skin predispose to colonization with potentially pathogenic bacteria, and subsequent detrimental effects on wound repair.

P0322
Priming through TLR4 but not TNFRs enhances inflammasome activation in human neutrophils

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Purpose/Objective: Objective: ATP is essential source of energy forcellular homeostasis.However, as a DAMP (damage associated molecular pattern) from injured or dying cells and tissues, ATP hasdeleterious effects on cells, including a variety of immune cells resulting in unwanted immune responses leading to the progression of tissue damage.ATP induced innate immune responses are mediated through NLRP3 inflammasome activation. However, the role of this process in neutrophils is not well explored. In the current study we investigate the effect of neutrophil priming in ATP induced inflammasome activation.
Materials and methods: Method: Primary neutrophils were isolated from whole peripheral human blood.TNF-alpha and TLR4 agonist (LPS) were used as priming agents prior to ATP exposure. IL-1beta level was measured by ELISA as an indicator of inflammasome activation andIL-8 level was measured as non-inflammasome mediated response.
Results: Results: LPS priming significantly increased ATP-inducedIL-1beta secretion by human neutrophils whereas TNF-alpha priming did not produce similar effects. Furthermore, IL-8 secretion induced by either TLR4 or TNFR stimuli was not influenced by inflammasome activation.
Conclusions:
Conclusion: Activation of TLR4 is a potent priming factor for inflammasome activation resulting in increased IL-1beta production whereas TNFR priming has little or no effect in human neutrophils.

P0323
The bacterial virulence factor TcpC activates the Nalp3 inflammasome by direct interaction

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Purpose/Objective: Toll-like receptors (TLRs) play essential roles in the activation of innate immune responses against microbial infections. They are characterized by an extracellular leucine-rich repeat and an

intracellular Toll/interleukin 1 receptor (TIR) domain. The TIR domain is crucial for TLR signaling by recruiting TIR containing adaptor proteins to the cytoplasmic domain of TLRs.

We recently described TIR domain containing proteins (Tcps) in the uropathogenic *Escherichia coli* strain CFT073 (TcpC) and in *Brucella melitensis* (TcpB) as a new class of virulence factors. Despite its inhibitory effect on TLR pathways TcpC is able to induce interleukin-1 beta (IL-1β) secretion suggesting a possible role of the inflammasome.
Materials and methods: The main focus of this work was to investigate the interaction between TcpC with components of the inflammasome. We therefore co-expressed TcpC in 293T cells together with various inflammasome associated proteins and performed immunoprecipitation experiments and confocal microscopy. Subsequent experiments using wild-type, ASC or Nalp3- deficient bone marrow derived macrophages (BMMs) infected with CFT073 addressed whether these inflammasome components are important for IL-1 β secretion.
Results: Indeed we observed an interaction or colocalization of a truncated form of TcpC with Nalp3 and Caspase-1. Support for a direct interaction of recombinant TIR-TcpC and Nalp3 was shown by an endogenous pull-down assay with lysates prepared of BMMs.

Infection experiments of BMMs with CFT073 revealed that TcpC-dependent induction of IL-1β secretion was strictly dependent on Nalp3 and ASC.
Conclusions: In summary our recent findings show, that TcpC inhibits TLRs but also activates the inflammasome.

P0324
The NLRP3 inflammasome in stable COPD

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Purpose/objective (introduction and objectives): The NLRP3 inflammasome is a multi-protein cytoplasmic sensor that governs the inflammatory response. Auto-inflammatory diseases are characterized by inflammasome abnormalities that result in excessive inflammation.

We hypothesized that the enhanced inflammatory response that characterizes chronic obstructive pulmonary disease (COPD) can have an auto-inflammatory component.

To test this hypothesis, we compared the expression, activity, functionality and transcriptional profile of the NLRP3 inflammasome in lung tissue samples and peripheral blood samples obtained from COPD patients, smokers (Sm) with normal spirometry and non-smoker (NSm) controls. Besides, we studied *in vitro* its functional response in isolated peripheral blood mononuclear cells (PBMCs).
Materials and methods: Immunohistochemistry techniques were used to assess the expression of caspase-1 in lung tissue, and Western blot, ELISA and/or real time PCR to quantify markers of inflammasome activation and related inflammatory mediators in lung tissue homogenates, serum and PBMCs *in vitro*.
Results:

1 Caspase-1, a core component of the NLRP3 inflammasome, was widely expressed in lung tissue.
2 Sm and COPD patients (including former Sm) had higher pulmonary protein levels of several makers of inflammasome activation, such as IL-1b, IL-1RA and IL-18. Besides, transcription of NLRP3 was increased in COPD patients, and mRNA levels of NLRP3 and IL-1b correlated negatively with the severity of airflow limitation.
3 Differences between groups were attenuated in serum, but serum caspase-1 levels were significantly related with those determined in lung tissue.
4 The *in vitro* functional response of the inflammasome in COPD was normal.

Conclusions: These results show that theNRLP3 inflammasome participates in the inflammatory response of COPD, exclude an auto-inflammatory component of COPD and support the use of serum caspase-1 levels as a systemic surrogate biomarker of inflammasome activation in the lung parenchyma.

P0325
The role of the NOD-like receptor NLRC3 in the innate immune system

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Purpose/Objective: The innate immune system enables eukaryotes to detect potential pathogenic elicitors in the host microbiome. It relies on several pattern recognition receptors (PRRs) to identify pathogen associated molecular patterns (PAMPs). One of the most prominent non-clonal, germline encoded but yet not fully characterized PRR group are the NOD-like receptors (NLR). In humans more than 20 NLRs have been identified and molecular evidence suggests that NLRs evolved to high pathogen specificity. NLRs occur in the closed boundaries of cells, upon detection of PAMPs they tend to oligomerize and form high molecular weight complexes. Those structures are an essential hub for further downstream signaling resulting in an inflammatory answer of the respective cell. Here, we aimed to analyze the expression pattern of NLRC3, a hitherto rarely characterized NOD-like receptor.
Materials and methods: In order to study the intracellular localization of NLRC3, we used immortalized HEK-293 and HeLa cells to overexpress NLRC3 and to conduct fluorescence staining. A tissue panel of human or murine origin was used to determine basal NLRC3 expression by real-time TaqMan PCR. Several stimulation regimes were applied. Western blotting using an antibody to NLRC3 was performed to confirm presence of NRLC3 in selected cell lines and tissues.
Results: We found NLRC3 to be strongly expressed in the cytosol of unstimulated epithelial HEK-293 cells and primary tissues. Especially immune related tissues like liver and spleen contain a high NLRC3 yield. Human peripheral blood mononuclear cells and murine bone marrow derived macrophages were found to be ideal models for stimulation experiments and expression analysis. Additionally we were able to show a close connection between the autoimmune disease Wegener's Granulomatosis and altered NLRC3 expression. In nasal mucosa biopsies from patients with Wegener's Granulomatosis the protein level of NLRC3 was significantly decreased compared to non-diseased individuals.
Conclusions: We identified epithelial and immune cells to constitutively express NLRC3. A disturbed NLRC3 expression pattern was shown in the nasal mucosa of Wegener's Granulomatosis patients, connecting NLRC3 to autoimmune diseases. Based on these findings, further studies will be employed to characterize NLRC3 function. Revealing the molecular function of NLRC3 will improve the understanding of the innate immune system.

P0326

Zinc-depletion activates processing and secretion of IL-1ß by inflammasome-dependent and independent mechanisms

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Purpose/Objective: Inflammation exacerbates the pathology of non-communicable disease. A key driver of this damaging inflammatory process is the pro-inflammatory cytokine interleukin-1beta (IL-1beta). IL-1beta is produced as an inactive precursor (pro-IL-1beta) that is cleaved by a protease called caspase-1 following its activation by the inflammasome complex. Multiple inflammasomes are known to exist but the best characterised, and the one implicated in sterile disease, is formed by the pattern recognition receptor NLRP3, the adaptor protein ASC and caspase-1. Zinc deficiency is a common cause or consequence of inflammatory disease and is prevalent in the aged population. The purpose of this study was to examine whether zinc depletion acted as an inflammatory stimulus to macrophages to induce the processing and release of IL-1beta via assembly of the NLRP3 inflammasome.

Materials and methods: Peritoneal macrophages were prepared from wild type (WT), NLRP3 and ASC knockout (KO) mice and cultured overnight. Cultures were then primed with bacterial endotoxin (lipopolysaccharide, LPS, 1 μg/ml) for 2 h before a 4 h treatment the zinc chelators TPEN or DTPA.

Results: Our data show that zinc depletion of LPS-primed macrophages induced the release of processed IL-1beta. Pharmacological studies using a cathepsin B inhibitor (Ca074), glyburide and a caspase-1 inhibitor (YVAD) suggested that zinc-depletion-induced IL-1beta processing was dependent on the NLRP3 inflammasome. However, studies in the knockout cells suggest that this is only partially true with multiple mechanisms of IL-1beta processing engaged.

Conclusions: These data suggest that zinc depletion can activate the processing of IL-1beta via multiple pathways. Zinc deficiency is a major contributor to inflammatory diseases of ageing and it is possible that the mechanisms we report here contribute to this effect.

Poster Session: Sensing – TLRs and Other Sensors

P0328

A naturally occurring carrier substance coupled to immunostimulatory RNA serves as a potent adjuvant delivery system

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Purpose/Objective: Vaccination is indispensable to prevent infectious diseases and therefore the development of safe and potent vaccine adjuvants becomes highly important. Here we investigate the specific activation of the innate immune system via TLR7 by coupling a TLR ligand to a natural carrier.
Materials and methods: Human PBMCs and murine Flt3L*induced mixed cultures of myeloid and plasmacytoid DCs of wt and TLR7$^{-/-}$ mice were used to analyze cytokine induction. These cells were incubated with immunostimulatory RNA coupled to the natural carrier versus complexation of the TLR7 ligand with the transfection reagent DOTAP. Supernatants were harvested after 20h and cytokine induction was analyzed by ELISA.

Murine Flt3L-induced cultures were incubated with fluorescent-stained RNA coupled to the natural carrier or complexed with DOTAP. Cellular uptake was analyzed by FACS and fluorescent microscopy.
Results: We could show that the TLR ligand coupled to the natural carrier is a potent inducer of IFNα and proinflammatory cytokines such as TNFα and IL-6. Interestingly, the TLR ligand coupled to the natural carrier leads to an increased IFNα secretion in comparison to complexation to the conventional transfection reagent DOTAP. Furthermore we were able to demonstrate that activation of the immune cells is strictly dependent on TLR7.

Coupling of the carrier to a non-stimulatory RNA did not lead to any cytokine induction which is an important aspect to prevent side-effects triggered by the carrier.

Cellular uptake of the TLR ligand coupled to the carrier was demonstrated by FACS-analysis and fluorescent microscopy.
Conclusions: In this study we developed a very potent adjuvant delivery system for TLR7. Our *in vitro* experiments showed an effective activation of immune cells stimulated with a TLR ligand coupled to the carrier. Thus, DOTAP induced side-effects could be abrogated by using this natural carrier. With focus on avoiding undesired side-effects like cell toxicity the use of a natural adjuvant delivery substance would be a major advantage for boosting immune responses initiated by vaccination.

P0329

Activated T cells detect intracellular single- and double-stranded DNA but fail to activate type I IFN responses – implication for restriction of HIV replication

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Purpose/Objective: During HIV replication the virus genome is both in RNA and DNA form. It is however unknown how or if they activate the innate immune reactions in T-cells. There are numerous classes of PRRs involved in recognition of viral specific components. This work is primary concentrated on cytosolic DNA receptors in T-cells. Interferon-inducible 16 (IFI16) has been described as an intracellular DNA sensor, that co-localize with the endogenous cytoplasmic DNA.
Materials and methods: PBMCs were isolated from buffy coats and stimulated with IL-2 and PHA. In this study we investigate which cytosolic DNA sensors, that recognize dsDNA in IL-2/PHA activated T-cells. These T-cells were transfected with labled ssDNA, dsDNA or ssRNA using the transfection agent Lipofectamine 2000. After 1 h of incubation, the cells were fixed and antibody stained for CD3, IFI16 and STING, which were visualized and quantified by confocal microscopy. After 6 h of incubation the mRNA level of IFN-β and CXCL-10 were measured by qPCR. Moreover the CXCL-10 protein level were measured by ELISA after 24 h of incubation.
Results: Transfected cytosolic DNA failed to stimulate anti-HIV activity in activated T cells. Flow-analysis confirmed efficient transfection. Confocal imaging showed that IFI16 relocated and co localized with transfected dsDNA and ssDNA, and not ssRNA. After DNA sensing in activated T-cells there was no activation of IFN responses.
Conclusions: We conclude that IL-2/PHA activated T-cells recognize cytosolic DNA, but fails to induce IFN responses. This data may provide insight into why T-cells are permissive to HIV infection.

P0331

Comparing the effects of human beta defensin 3 and LL-37 on Toll-like receptor signaling

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Purpose/Objective: Beta-defensins are key players in innate and adaptive immunity and recently human beta defensin-3 (hBD3) has been shown to limit production of pro-inflammatory cytokines in TLR4-stimulated macrophages. The MyD88 and TRIF pathways are both inhibited by hBD3, however the direct targets are yet to be identified. The human cathelicidin LL-37 has been shown to both augment and mitigate signaling by various Toll-like receptors and may drive autoimmune disease, such as psoriasis, by converting self-DNA into an effective activator of TLR9 in pDCs. This study compares the effects of hBD3 and LL-37 on stimulation of Toll-like receptors 2, 3, 7 and 9.
Materials and methods: Primary macrophages and pDCs were treated with with the TLR2, 3, 4, 7 or 9 agonists, Pam3CSK4, poly I:C, LPS, R848 and CpG respectively, in the presence or absence of hBD3 or LL-37. Cytokine levels in cell culture supernatants were measured by ELISA.
Results: We show that although both hBD3 and LL-37 suppress and increase particular TLR signaling pathways, they also demonstrate distinct effects. hBD3 and LL-37 suppress LPS-induced TNF alpha, IL-6 and RANTES, however neither have an effect on cytokine induction by TLR2 or TLR7. In addition, hBD3 and LL-37 exacerbate the proinflammatory effect of the TLR3 agonist pI:C. In contrast, IFN alpha induction by CpG is amplified by the presence of LL-37 but inhibited by the presence of hBD3. Furthermore, hBD3 abolishes this LL-37-enhanced TLR9 activation in pDCs.
Conclusions: We have shown that hBD3 interacts with TLR signaling pathways to both suppress and augment cytokine and interferon production. hBD3 is part of a cluster of highly copy number variable genes and individuals may have variable expression levels, the effects of which may have implications for autoimmune disease. In addition the enhanced effects of hBD3 and LL-37 on pI:C should be considered in the development of nucleic adjuvants in vaccines.

P0332

Expression of melatonin receptors in the cells of immune system

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Purpose/Objective: Melatonin is a hormone that regulates many physiological circadian rhythms. It regulates and controls biological clock, stimulates the immune system and protects the central nervous system. The disrupted synthesis of melatonin leads to pathological process, including cancer, autoimmune diseases; i.e. diseases caused by the disorder of the immune system.

Melatonin acts via four types of receptors: MT1, MT2, MT3 and nuclear receptors (ROR*a*, RZRβ, and RORγ). The knowledge about the expression of melatonin receptors on the cells of the immune system is insufficient. The data about detail expression of those receptors could contribute to understand the mechanisms of the immunomoduliatory action of melatonin.

Materials and methods: We analysed the expression of melatonin receptors in the murine organs (spleen, bone marrow, thymus, lymph nodes) and cells (macrophages, dendritic cells, granulocytes) of immune system. The analysis was performed using rtPCR.

Results:

The main research findings include: mRNA of MT1 melatonin receptor was found mainly in the thymus. A weak expression of MT1 was detected in the granulocytes. MT1 was not detected in the bone marrow, lymph nodes, spleen, macrophages and dendritic cells.

MT2 mRNA was found in the thymus only. It was not detected in the bone marrow, lymph nodes, spleen, granulocytes, macrophages and dendritic cells.

RORg mRNA was detected in the thymus and granulocytes. Also, we amplified very small amounts of RORg mRNA in the lymph nodes and bone morrow cells. However, this receptor was not detected in spleen, macrophages and dendritic cells.

MT3 mRNA was found in all tested organs (spleen, bone marrow, thymus, lymph nodes) and cells (macrophages, dendritic cells, granulocytes).

RORa mRNA was found in all tested organs (spleen, bone marrow, thymus, lymph nodes), in the macrophages and dendritic cells. However, this receptor was not detected in granulocytes.

RORb mRNA was not detected in all tested organs and cells.

Conclusions: During research work was investigated the expression of melatonin receptors in the murine organs (spleen, bone marrow, thymus, lymph node) and cells (macrophages, dendritic cells, granulocytes) of immune system.

P0333

Extracellular toll like receptor regulator RP105/CD180 ameliorates atherosclerosis via its role on B cells

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Purpose/Objective: RP105 is a structural homolog of TLR4 and an important regulator of TLR4 signaling. RP105 enhances B-cells activation while it dampens the TLR4 response on myeloid cells. B-cells are known to be important in autoimmune disease and increase atherosclerosis independently of antibody production or presence of other leukocytes. RP105 is also linked to autoimmune disease however a role for RP105 in atherosclerosis still remains unknown.

Materials and methods: Male LDLR$^{-/-}$ mice were irradiated and subsequently received bone marrow from either RP105$^{-/-}$ or wild type control mice. After 6 weeks of recovery the mice were fed a hypercholesterolemic diet for 9 weeks and sacrificed for further analysis.

Results: Plaque burden in the proximal aorta was significantly attenuated ($230 \pm 26 \times 10^3$ μm^2 versus $131 \pm 15 \times 10^3$ μm^2 $P = 0.004$) in the RP105$^{-/-}$ chimeras. No difference in activation of Dendritic Cells, Macrophages, or T cells was found whereas B cell activation was significantly reduced in the RP105$^{-/-}$ chimeras as depicted by the percentage of cell surface bound IgM and CD86 expression on CD19$^+$ B cells. Expression of pro-inflammatory cytokines was significantly decreased in the spleen of RP105$^{-/-}$ chimeras. IgM against oxLDL or MDA-LDL were not altered while IgG levels against oxLDL and MDA-LDL were significantly lowered in the RP105$^{-/-}$ chimeras. *In vitro* B cells from RP105$^{-/-}$ mice showed reduced activation and proliferation upon LPS stimulation but not upon αCD40 stimulation and a decrease in IL6 production. Furthermore *ex vivo* stimulation showed that splenocytes from RP105$^{-/-}$ chimeras showed less proliferation upon oxLDL or LPS incubation. Although we could not find a difference in systemic T-cells activation, we did find a significant reduction in both intimal and perivascular T lymphocyte and macrophage accumulation.

Conclusions: Our study is the first to show a TLR mediated role for B cells in atherosclerosis via the effects of RP105 in an antibody and cytokine dependant manner.

P0334

Functional and biological characterization of SSc5D, a novel molecule of the scavenger receptor cysteine−rich family

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Purpose/Objective: SSc5D is a member of the Scavenger Receptor Cysteine-Rich (SRCR) superfamily. This group of proteins is characterized by the presence of one or more SRCR domains, which are thought to mediate protein-protein interactions within the immune system, or to serve as pathogen pattern recognition motifs. SSc5D consists of two structurally distinct parts − a N-terminal domain containing five SRCR domains, and a C-terminal domain made up of a high number of repetitive and extensively O-glycosylated sequences similar to mucins. The overall function of the SRCR family is still poorly understood; therefore unraveling the biological and functional properties of SSc5D may help to clarify the role of this group of highly conserved proteins.

Materials and methods: SSc5D cell expression at the mRNA and protein levels was analyzed in different cells and cell lines, and tissue-expression analysis was accessed by immunohistochemical staining of normal and pathological sections of uterus, placenta, spleen, kidney, etc. To evaluate the binding of SSc5D to different types of cell, including different pathogens, the two distinct domains of SSc5D* the SRCR containing domain, and the mucin-like domain − were independently produced.

Results: SSc5D is highly-expressed in PBMCs, in particular in CD4$^+$ T lymphocytes and in the monocytic lineage cells. Nevertheless, it is also detected in other cell-types from different tissues, although presenting different molecular weights. This suggests that post-transcriptional or -translational modifications may be cell-specific. Confirming our previous results from Northern blotting analysis, a high expression of SSc5D in placenta was detected by immunohistochemical staining of sections, where we observed the localization of SSc5D in syncytiotrophoblast cells, which can suggest a possible role in fetal immunity.

Conclusions: SSc5D is expressed in different types of cells and tissues and its peculiar characteristics suggest a role as a novel immune receptor. Ours and other's results suggest that SSc5D may be produced by a variety of different cells and provide a molecular bridge for the interaction of immune cells with pathogens in different tissues.
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P0335

Functional significance of evolutionary divergence in Toll-like receptor-regulated gene expression in human versus mouse

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Purpose/Objective: Host immune response genes must co-evolve with rapidly evolving pathogens. Thus, species-differences in immune genes are likely to be particularly important in host defence. Differences in the way that orthologous genes are expressed are thought to be particularly critical for phenotypic differences between species. This study defined the extent of divergence in TLR4-regulated gene expression between primary human and mouse macrophages, the underlying mechanisms responsible for this divergence, and the functional significance of 'human-specific' TLR4-target genes in host defence.
Materials and methods: A custom expression profiling platform was developed to compare TLR4 regulated expression of orthologous genes in human versus mouse. Species-specific gene regulation was confirmed across a panel of human and mouse macrophage cell types at the mRNA and protein level. Promoters of TLR4 target genes (identified on a genome wide-scale) were interrogated to identify mechanisms of regulatory divergence. Functional analysis of human-specific TLR4 target genes was performed in primary human macrophages responding to Gram-negative bacterial pathogens.
Results: Although there was a pattern of conservation in TLR4-regulated gene expression between human and mouse, approximately 7.5% of TLR4 target genes showed striking divergence in regulation between these species across an LPS time course. Features of divergently regulated gene promoters include TATA box enrichment, CpG island depletion and, paradoxically, enhanced sequence conservation. Divergence in anti-microbial effector genes was common. Functional analysis of 'human-specific' TLR4 target genes identified a role for a novel zinc transporter in human macrophage anti-microbial responses, since knock-down of this gene in primary human macrophages impaired clearance of intracellular *E. coli*, whilst exogenous zinc enhanced killing of *E. coli*. However, this transporter has been hijacked by *Salmonella typhimurium* to permit intracellular survival of this pathogen.
Conclusions: Striking divergence in the regulation of innate immune genes is common. Highly conserved, complex promoters control the expression of these genes. This complexity likely increases their susceptibility to evolutionary change. Many 'human-specific' TLR4 target genes are likely to have important, unexplored roles in host defence.

P0336

Gene expression induced by Toll-like receptors in macrophages requires the transcription factor NFAT5

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Purpose/Objective: Toll-like receptors engage networks of transcriptional regulators to induce genes essential for antimicrobial immunity. The map of transcription factors downstream TLRs is not fully understood and our work identifies NFAT5 as a novel regulator of mammalian anti-pathogen responses downstream TLRs.
Materials and methods: We used bone marrow derived macrophages (BMDMs) and peritoneal macrophages from WT and NFAT5$^{-/-}$ mice stimulated with LPS from *E. coli*. Gene expression and regulation of transcription was assessed by western blot, RT-qPCR, cDNA microarray analysis and Chromatin Immunoprecipitation assays. Luciferase reporter assays and siRNA technology was also used to further study gene expression regulation. As *in vivo* models, we have used LPS injection and *Leishmania major* infection.
Results: We show that upon treatment of macrophages with TLR agonists, NFAT5 is induced and recruited to the regulatory regions of pro-inflammatory genes. Accordingly, NFAT5-deficent bone marrow derived macrophages (BMDMs) show a selective defect in the expression of pro-inflammatory and antimicrobial genes such as *Nos2*, *Il6* and *Tnf*. Moreover, we show that NFAT5 exhibits two modes of association with its target genes, as it is constitutively bound to *Tnf* and other genes, while its recruitment to *Nos2* or *Il6* requires TLR activation. Further analysis revealed that TLR-induced recruitment of NFAT5 to *Nos2* is dependent on IKKβ activity, *de novo* protein synthesis and sensitive to histone deacetytases. cDNA microarray analysis allowed us to determine to what extent NFAT5 contributes to the inflammatory gene expression program. Consistent with our *in vitro* studies, peritoneal macrophages from NFAT5$^{-/-}$ mice challenged *in vivo* with LPS displayed reduced expression of several pro-inflammatory and anti-microbial genes compared to their wild type littermates. Moreover, experiments of infection of mice with *Leishmania major*, demonstrated that NFAT5 is a key factor in the control of *L. major* replication and dissemination.
Conclusions: These findings unravel new aspects on the mechanisms regulating the cellular response to microbial products and provide important new insights into the regulation of inflammatory diseases. Altogether this work identifies NFAT5 as a novel regulator of mammalian anti-pathogen responses.

P0337

Genetic screens in evolutionary divergent wild-derived mice reveal novel regulators of the balance between inflammation and cell death

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Purpose/Objective: Using evolutionary divergent wild-derived mice, we identified several molecular differences between 'wild-derived' inbred strains of mice (such as MSM and MOLF/Ei) and classical inbred strains (such as C57BL6) at the IRAK2 locus, which lead to potent acute phase innate immune responses in wild-derived mice. In addition, we identified a novel anti-inflammatory function of IRAK1bp1. Here we report a novel role for CYLD in maintaining the balance pro-inflammatory signaling and cell-death.
Materials and methods: Peritoneal macrophages from B6 and MOLF were stimulated with LPS or IMQ$^{+/-}$ zVAD$^{+/-}$Nec-1 and assayed for

viability.Each mouse from the panel of backcross (B6 × B6 × MOLF) mice was genotyped using microsatellite markers.QTXb analyzed d the correlation between genotype and phenotype at each marker.Likelihood of odds (LOD) score measures the likelihood that phenotype is linked to genotype at a given locus.LOD>3 is significant.C. Genotype of N2 mice at D8Mit163 (within the peak of highest linkage).Viability phenotype at D8Mit163 is associated with more viability following IMQ^{+}zVAD stimulation.

Results: During ell death through RIP1-mediated regulated necrosis is emerging as an important mechanism through which cells respond to certain viral pathogens. To determine novel regulatory mechanisms of this signaling pathway, we studied cellular responses to Toll-like receptor (TLR) agonists in evolutionarily divergent wild-derived mice (MOLF/Ei) in a forward genetic screen of TLR-induced necrosis. In contrast to C57BL/6 macrophages, which are susceptible to TLR-induced necrosis, MOLF macrophages are resistant to death induced through this pathway. Using an N2 panel and forward genetic analysis, we found a locus on chromosome 8 that confers resistance to TLR-induced necrosis. Through gene expression analysis, allelic bias studies, and siRNA knockdown in peritoneal macrophages, we concluded that a genetic difference in CYLD confers the differential susceptibility to TLR-induced necrosis between B6 and MOLF macrophages.This difference was revealed through analysis of the regulation of CYLD mRNA species following TLR stimulation.The molecular basis for in greater survival of MOLF cells appears to be down-regulation of full-length CYLD mRNA following activation.

Conclusions: Through our use of wild-derived mice in forward genetic analysis, we were able to uncover a novel regulatory mechanism CYLD in a physiologic setting.

P0338

Hepatic regulation of pro-inflammatory stimuli through communication of liver sinusoidal endothelial cells and hepatocytes

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Purpose/Objective: In the liver, hepatocytes are in close contact with liver sinusoidal endothelial cells (LSECs). These LSECs separate hepatocytes from leukocytes passing through the liver sinusoid. Although LSECs are known to be fenestrated and not interconnected by tight- junctions, they have been shown to form a functional barrier between hepatocytes and circulating leukocytes. LSECs and hepatocytes therefore fulfill different, yet complimentary functions: hepatocyte exert mainly metabolic functions whereas LSECs possess extraordinary scavenger functions. Distinct and mutually exclusive expression of different receptors and regulating molecules forms the molecular basis for cooperative and instructive interaction between these two cell populations. Ideally, the interaction of hepatocytes and LSECs is aimed at optimal functional adaptation.

Materials and methods: Presence of defined proinflammatory pathogen–associated molecular patterns (PAMP) is sensed through germ–line encoded pattern recognition receptors (PRRs), the majority of which are Toll–like Receptors (TLRs).

Results: We have been able to define the molecular basis for PAMP–recognition on LSECs. Here, we show new insights of the communication between hepatocytes and LSECs, which are crucial for the regulation of biological functions in hepatocytes by signalling of distinct TLRs and the expression of proinflammatory cytokines in LSECs.

Conclusions: Thus, we define the molecular basis how LSECs integrate and present microenvironmental signals to hepatocytes, thereby extrapolating signalling processes from the level of sinusoidal compartment into the whole organ.

P0339

Identification of a novel IFN-inducible short isoform of RIG-I that acts as feedback negative regulator

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Purpose/Objective: Innate immune signaling in response to viral infection is triggered by differential activation of the cytosolic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). Activation of these receptors leads to the induction of type I interferon (IFN I) and proinflammatory cytokines. RIG-I is the prototypic member of the RLR family. Therefore, the mechanisms of RIG-I activation and its modulation to prevent uncontrolled IFN I production were extensively studied. In line with this, we identified a novel isoform of human RIG-I that acts as inhibitor of its own pathway.

Materials and methods: RIG-I isoform expression was monitored by western blot. Identification of the newly identified RIG-I variant (RIG-I SF) was assessed by immunoprecipitation and mass spectrometry analysis. To illuminate the function of RIG-I SF, the corresponding sequence was cloned and luciferase reporter assay, ELISA and site-directed mutagenesis were applied. Proteasome and caspase-specific inhibitors were tested to investigate the generation of the novel RIG-I variant.

Results: Detection of endogenous RIG-I in WCLs of human cells after IFN I stimulation revealed a yet unknown short 95 kDa isoform of RIG-I. RIG-I SF is expressed in a time and dose-dependent manner in response to IFN I treatment, pppRNA transfection and virus infection, with a delayed kinetics compared to RIG-I splice variant. Mass spectrometry analysis indicated that RIG-I SF might comprise amino acids L155 to K925 of RIG-I. Overexpression of RIG-I SF leads to a dose-dependent inhibition of the RIG-I-mediated IFN response. This inhibitory capacity was absent when RIG-I SF bearing point mutations in the pppRNA-binding site was analyzed. Analysis of WCLs of cells pretreated with proteasome or caspase inhibitors indicated the involvement of the proteasome in the generation of RIG-I SF.

Conclusions: We identified a new IFN inducible isoform of RIG-I, which is structurally different from RIG-I splice variant and generated by proteasomal cleavage. RIG-I SF acts as negative regulator of its own pathway and seems to exert its regulatory function by competitive binding of the RNA ligand. We hypothesize that RIG-I SF is involved in controlling the activation of endogenous RIG-I in infected cells, thereby balancing the innate immune response.

P0340

IgG opsonization of bacteria promotes T helper 17 responses via synergy between Toll-like receptors and Fc gamma receptor IIa in human dendritic cells

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Purpose/Objective: Dendritic cells (DCs) are essential in inducing adaptive immunity against bacteria by expressing cytokines that skew towards protective T helper 17 (Th17) cells. Although it is widely recognized that induction of these cytokines by DCs involves activation of multiple receptors, it is still incompletely characterized which combination of receptors drives Th17 cell responses upon exposure to

bacteria. In the present study we have taken into account that in most conditions DCs will engage bacteria opsonized by cross-reactive IgG, resulting in simultaneous stimulation of Fc gamma receptors (FcγRs) and bacterial sensors. Therefore, the objective of this study was to determine how IgG opsonization of bacteria affects T helper cell polarization and subsequently to unravel the underlying mechanisms.
Materials and methods: Human monocyte-derived DCs were stimulated with IgG-opsonized bacteria or specific Toll-like receptor (TLR) ligands and complexed IgG. Cytokines were measured both at mRNA and protein level, and caspase-1 activation using a FLICA-assay. Th17 induction was assessed by measuring IL-17 production after DC-T cell co-culturing.
Results: IgG opsonization of bacteria strongly promoted Th17 responses by DCs, which was FcγRIIa-dependent and coincided with enhanced production of selected cytokines by DCs, including Th17-promoting IL-1β and IL-23. Notably, FcγRIIa stimulation on DCs did not induce cytokine production when stimulated individually, but selectively amplified cytokine responses through synergy with TLR2, 4 or 5. Importantly, this synergy is mediated at two different levels. First, TLR-FcγRIIa co-stimulation strongly increased transcription of pro-IL-1β and IL-23p19. Second, FcγRIIa triggering induced activation of caspase-1, which cleaves pro-IL-1β into its bioactive form and thereby enhanced IL-1β secretion.
Conclusions: Here we identified IgG opsonization of bacteria as a new mechanism by which the immune system promotes Th17 responses. IgG opsonization promotes Th17 induction by DCs, which fully depends on synergy between TLRs and FcγRIIa. Since most tissues just below the epithelial barriers contain abundant amounts of DCs and interstitial IgGs, this may be a universally relevant natural mechanism to counteract bacterial infections in numerous sites in the human body.

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P0341
Modulation of the inflammatory response in gestation associated tissues by IL-4

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Purpose/Objective: Preterm birth (PTB) is the leading cause of perinatal morbidity and mortality in the Western world. Local and systemic inflammation is a feature of preterm labour and delivery. Inflammatory mediators such as IL-1β and MIP-1α are produced by the three main gestation-associated tissues (amnion, choriodecidua and placenta) in response to pathogen-associated molecular patterns (PAMPs).The cytokines interleukin (IL)-4, IL-10 and IL-13 are anti-inflammatory and might have potential as anti-inflammatory therapies to prevent PTB.
Materials and methods: The effect of IL-4 (10 ng/ml) on gestation-associated tissues was determined in response PAMPs; LPS (10 ng/ml), Pam3CSK4 (100 ng/ml) and FSL-1 (10 ng/ml). Tissue explants were pre-stimulated with IL-4 for 90 min, prior to PAMP stimulation for 24 h. Tissue free supernatants were assayed for IL-1β and MIP-1α levels by ELISA. IL-4 induced STAT6 phosphorylation was also examined by immunoblotting.
Results: IL-4 reduced IL-1β and MIP-1α production in term healthy placentas in response to all PAMPs studied. The percentage reduction compared to PAMP alone was for: LPS (IL-1β, 91%, mean [86−99%, range], $P = 0.002$; MIP-1α, 86% [83−89%], $P = 0.001$), Pam3CSK4 (IL-1β, 83% [78−86%], $P = 0.001$; MIP-1α 72% [62−91%], $P = 0.001$), and FSL-1 (IL-1β, 74% [43−91%], $P = 0.04$; MIP-1α, 95% [93−96%] $P < 0.001$). STAT6 phosphorylation in response to IL-4 was demonstrated in the placenta. The effect of IL-4 on the response to PAMPs by the amnion and choriodecidua was less consistent; IL-4 only reduced IL-1β and MIP-1α production in response to Pam3CSK4 [choriodecidua (IL-1β, 51% [32−58%], $P = 0.005$; MIP-1α, 36% [25−48%] $P = 0.035$), amnion (IL-1β 72% [0−44%] $P = 0.011$; MIP-1α, 48% [36−63%], $P = 0.017$)].
Conclusions: IL-4 can reduce the inflammatory response primarily in the placenta and to a lesser extent in the amnion and choriodecidua. Greater understanding of the IL-4 signalling pathway might reveal novel approaches for the development of therapeutics for treating women in preterm labour.

P0342
Neutralization of pathogenic Gram-positive bacteria by macrophages is independent from TLR2, -3, -4, -7, and -9 yet depends upon MyD88

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Purpose/Objective: Blockade of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) has been suggested by us and others as a strategy to inhibit sepsis pathology upon Gram-negative bacterial infection. Gram-positive bacterial infection, however, is also a major cause of septic shock but was recognized even by macrophages that lacked expression of functional TLR2, -3, -4, -7, and -9. However, macrophages lacking MyD88 were unresponsive to Gram-positive bacterial challenge.
Materials and methods: The read out of cellular bacteria recognition was Gram-positive bacteria counts in macrophage culture supernatants. Specifically, we quantified bacteria upon infection to monitor biocidal macrophage activity.
Results: That Gram-positive bacteria recognition was independent of TLR2 was surprising given the central role in Gram-positive bacteria recognition that has been assigned to TLR2 by the literature. Own findings and further literature, however, implied Gram-positive bacterial RNA as important PAMP. Therefore, we fractionate total RNA to narrow down the responsible RNA subpopulation and to characterize Gram-positive bacteria-driven cell activation in further detail.
Conclusions: Potentially, our results will point at novel means for interrupting pathogenesis toward septic shock in Gram-positive bacterial infection.

P0343
Novel functions of a regulator of lysosomal trafficking, LYST in TLR4 signal transduction

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Purpose/Objective: Toll-like receptors (TLRs) play a critical role in the recognition of microbial components in innate immune cells. Toll-like receptor 4 (TLR4) recognizes lipopolysaccharide (LPS) from Gram-negative bacteria and resent studies suggest that TLR4 is able to activate signaling pathways at endolysosomal compartment after

receptor internalization. However, little is known about the regulatory mechanisms of endolysosomal signaling mediated through TLR4 and if this pathway serves important protective functions for bacterial infections.

LYST is a regulator of lysosomal trafficking and mutations in the LYST gene cause the Chediak-Higashi syndrome (CHS) in humans and the phenotype of the beige-mouse (Kaplan et al., 2008). A prominent feature of this disease is the accumulation of enlarged lysosome-related granules in a variety of cells. CHS patients suffer from recurrent bacterial infections, however, the regulatory mechanism underlying LYST mediated anti-bacterial immunity is largely unclear. Hence, the objective of the study was to investigate the role of LYST in TLR4 signaling pathways and in the immune response against Salmonella infection.

Materials and methods: Utilizing a genetic system of LYST-deficient mice our study followed a multidisciplinary approach, which involved biochemical, cell biological and immunological methods to analyze the impact of lysosomal trafficking regulator LYST on TLR4 signaling and protective immunity against Salmonella infection.

Results: We observed that Lyst deficient cells exhibit defective TLR signaling, specifically in TLR3 and TLR4 pathways, but intact signaling through other TLRs, such as TLR2, TLR5, TLR7 and TLR9. Importantly, this correlated with significantly impaired immunity against *Salmonella* infection in Lyst-mutant *beige* mice. Also, in *in vivo* studies LYST-mutant *beige* mice exhibited a significantly reduced ability to produce inflammatory cytokines in response to LPS. Subsequent mechanistic *in vitro* experiments revealed impaired IRF3 phosphorylation in LYST mutant macrophages upon LPS stimulation, suggesting that LYST is involved in the control of endolysosomal signaling of TLR4, which activates the TRIF signaling pathway.

Conclusions: Our studies provide evidence for a so far unrecognized function of LYST as a linker between lysosomal function and TLR4 signaling.

P0344

Paracoccidioides brasiliensis and mannan use MR, CR3 and TLR4 to interact with macrophages but induce opposite activation profiles in macrophages of resistant and susceptible mice

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Purpose/Objective: In addition to alpha1,3 glucan, mannan and mannan-linked proteins are expressed in the outer layer of *Paracoccidioides brasiliensis* yeasts. Mannosyl residues are recognized by several membrane pathogen recognition receptors (PRRs), such as the mannose receptor (MR), complement receptor 3 (CR3) and Toll-like receptor 4 (TLR4) on macrophage membranes.

Materials and methods: The aim of this study was to clarify the role of these receptors in the interaction between *P. brasiliensis* and macrophages from resistant (A/J) and susceptible (B10.A) mice. In addition, the mRNA expression of SOCS3, a protein associated with M1 macrophages; was also evaluated.

Results: We verified that mannan and anti-MR antibody exert antagonistic effects, the former increasing and the latter decreasing the killing ability and nitric oxide production of macrophages. In addition, the specific blockade of MR, CR3 and TLR4 impaired fungal recognition and modulated the production of cytokines. Interestingly, both mannan and *P. brasiliensis* induced the production of IL-12 by B10.A macrophages, whereas TNF-α, IL-6 and TGF-β were produced by A/J cells. Normal B10.A and A/J macrophages expressed different levels of phagocytic and non-phagocytic PRRs. Mannan and *P. brasiliensis* infection led to decreased expression of MR and TLR2 on A/J macrophages, whereas CR3, TLR4 and TLR2 were reduced on B10.A cells. Accordingly the expression of SOCS3 mRNA was significantly higher in B10.A macrophages indicating a preferential M1 profile.

Conclusions: In conclusion, macrophages appear to use MR, CR3 and TLR4 to interact with *P. brasiliensis*; however, the resulting divergent activation profiles appear to be dependent on the different expression and engagement of PRRs by genetically distinct cells.

P0345

Platelets express functional IL-1R family members

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Purpose/Objective: TIR8 is a member of the IL-1R like (ILR) family, which acts as a negative regulator of TLR signalling playing non-redundant roles in avoiding inappropriate inflammatory responses in pathological conditions, including infections, colitis, autoimmunity and cancer.

Growing evidence indicates that LPS-mediated signal via TLR4 leads to platelet activation, platelet-leukocyte interaction and neutrophils extracellular trap (NETs) production in severe sepsis. These data prompted us to investigate the expression and function of other IL1R/TLR family members on platelets to better understand the complex interaction between leukocytes and platelets.

Materials and methods: TIR8, IL1R and IL18R expression and CD62P modulation was evaluated by FACS and confocal microscopy analysis.

Thrombus formation in $TIR8^{-/-}$ murine model was evaluated in CCl_4 induced acute inflammation by IHC analysis.

Results: We first investigated the expression of human TIR8 on platelets and megakaryocytic cell lines. Interestingly, we found that platelets and their precursors showed higher surface expression level as compared to other leukocytes. We next studied the expression of IL1R/TLR family members and their function in response to specific ligands. Data obtained showed that TIR8 as well as IL1RI and IL18R are expressed by human and murine platelets and megakaryocytic cell lines and that TIR8 surface expression is modulated by IL18, IL1b and LPS on megakaryocytic cell lines, involving dense-like granule trafficking. Moreover in patients with Systemic Inflammatory Response Syndrome (SIRS) TIR8 surface expression was downmodulated as compared to age matched healthy donors.

IL18R, IL1R and TLR4 activation induced P-selectin upregulation on both human and murine platelets surface and a dysregulated ADP-mediated aggregation, supporting a role of these receptors on the thrombogenic process.

Finally we used a genetic approach to investigate the role of TIR8 on platelet activation. Data obtained so far suggest that TIR8 is involved in platelet activation and liver sinusoid thrombus formation in response to CCl_4 induced acute inflammation.

Conclusions: Our results show that besides TLR family members, platelets also express functional ILR members, including IL1RI and IL18R and TIR8, and suggest a novel role for TIR8 and ILR members in the regulation of platelet activation.

P0346

Poly (I:C)-induced CXCL10 production in adrenocortical cells – implications for autoimmune Addison's disease?

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Purpose/Objective: Autoimmune Addison's disease (AAD) is caused by selective destruction of the steroid hormone-secreting cells of the

adrenal cortex. While T cells are thought to be key players in this autoimmune attack, the actual role played by the target organ has been somewhat overlooked. Adrenocortical cells have been shown to produce CXCL10 when exposed to pro-inflammatory cytokines, and this chemokine is found to be elevated in sera from AAD patients compared to healthy controls. As the adrenal cortex is permissive to certain viruses and bacteria, which may be sensed by Toll-like receptors (TLRs), we hypothesized that certain TLR ligands could also stimulate CXCL10 production in adrenocortical cells.

Materials and methods: Adrenocortical cells were stimulated with poly (I:C), a substitute for viral dsRNA, alone or in combination with pro-inflammatory cytokines. TLR3 expression in adrenocortical cells was evaluated by indirect immunofluorescence.

Results: We found that poly (I:C) stimulated CXCL10 secretion from adrenocortical cells in a TLR3-dependent manner. Furthermore, we observed a synergistic effect of pro-inflammatory cytokines and poly (I:C) on CXCL10 production.

Conclusions: The present findings could represent a potential link between viral infections of the adrenal cortex and AAD, wherein viral dsRNA and pro-inflammatory cytokines could induce the secretion of T-cell-recruiting CXCL10 from adrenocortical cells and thus drive the infiltration of auto-reactive T cells into the adrenal cortex. Future studies should therefore focus on which viral strains that could induce production of CXCL10 in adrenocortical cells and whether such chemokine secretion actually is chemotactic for auto-reactive T cells in AAD.

P0347

Pre-reperfusion treatment with OPN-305, a humanized anti–Toll-like receptor-2 antibody, reduces myocardialischemia/reperfusion injury in pigs

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Purpose/Objective: Toll-like receptor 2 (TLR2) is an important mediator of innate immunity and ischemia/reperfusion-induced injury. We have previously shown that TLR2 inhibition reduces infarct size and improves cardiac function in a mouse model. However, the therapeutic efficacy of a clinical grade humanized anti-TLR2 antibody, OPN-305, in a large-animal model remained to be addressed.

Materials and methods: Pigs underwent 75 min ischemia followed by 24 h of reperfusion. Saline or OPN-305 (25, 12.5, 6.25, or 1.56 mg/kg) was infused intravenously 15 min before reperfusion. Cardiac function and geometry were assessed by echocardiography. Infarct size was calculated as the percentage of the area at risk and cardiomyocyte damage was assessed biochemically by serum Troponin-I concentrations.

Results: Flow cytometry analysis revealed specific binding of OPN-305 to porcine TLR2. *In vivo*, OPN-305 exhibited a secondary half-life of 8 days. Intravenous administration of OPN-305 before reperfusion significantly reduced infarct size (45% reduction, $P = 0.041$) in a dose-dependent manner. In addition, pigs treated with OPN-305 exhibited a significant preservation of systolic performance in a dose-dependent fashion, whereas saline treatment completely diminished the contractile performance of the ischemic/reperfused myocardium. OPN-305 has completed Phase I trials in healthy human volunteers (HV) and the PK/PD has been established. Treatment of HV with OPN-305 led to a significant decrease in IL-6 secretion post TLR2 agonist stimulation, which correlated with receptor occupancy and PK.

Conclusions: OPN-305 significantly reduced infarct size and preserved cardiac function in pigs after ischemia/reperfusion injury. Hence, OPN-305 is a promising adjunctive therapeutic for patients with acute myocardial infarction.

P0348

Prognostic value of the presence and expression of TLR7 in non small cell lung carcinoma (NSCLC) patients

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Purpose/Objective: Lung cancer accounts for over one million deaths/year with a 5-year survival of 8–12%. TLR7 detects Pathogen- and Damaged-Associated Molecular Patterns triggering inflammatory responses with the production of proinflammatory cytokines and interferons. In a recent study we demonstrated the expression of TLR7 in primary NSCLC and observed that stimulation of cell lines with TLR7 agonist lead to the upregulation of antiapoptotic protein Bcl-2 and tumor cell survival and chemoresistance [1].This suggests an important role of TLR7 in tumor progression. In this study we thus wanted to correlate the expression of TLR7 with prognostic value among a cohort of NSCLC patients.

Materials and methods: We performed immunohistochemical staining of TLR7 on 278 paraffin embedded NSCLC samples.

Results: TLR7 expression was heterogeneous with 65% ($n = 181$) of the patients being TLR7 positive and 45% ($n = 97$) TLR7 negative. Overall survival was determined by Kaplan-Meier curves and log-rank test. At an optimal cutoff of 82% we found 21% ($n = 58$) of all the patients (stage I to III) had high expression of TLR7 (>82%) on the tumoral cells with a bad prognosis compared to the TLR7 low group ($P = 0.002$). A highly significant poor prognosis among the TLR7 high group was observed compared to the low group for the stage III patients ($P = 0.0003$). There was a significant difference between TLR7 high and low groups for adenocarcinoma patients ($P = 0.0031$) but no difference for the patients with squamous cell carcinoma.

Conclusions: These results suggest a bad prognostic role of TLR7 expression on tumoral cells in adenocarcinoma NSCLC patients.

As we demonstrated for the first time that TLR7 is involved in chemoresistance, we hypothesize that the multi drug resistance genes maybe upregulated in TLR7 expressing cells. We are performing gene profiling assays on sorted tumors to look for molecular markers that could be implicated in chemoresistance due to TLR7 stimulation.

[1] Cherfils-Vicini, J, et al. Triggering of TLR7 and TLR8 expressed by human lung cancer cells induces cell survival and chemoresistance. J Clin Invest. 1;120(4):1285–97, 2010

P0349

Role of TLR4 on recruitment/activation of microglia in the hypothalamus during obesity

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Purpose/Objective: TLR4-dependent hypothalamic inflammation is a common feature of experimental diet-induced obesity. Long-chain saturated fatty acids present in the diet activate TLR4 signaling in microglia cells leading to the expression of inflammatory cytokines and subsequent induction of leptin resistance in hypothalamic neurons involved in the control offood intake and energy expenditure. It is currently unknown if diet-induced hypothalamic inflammation is dependent on bone-marrow (BM) derived cells or only in resident microglia. Here, we investigate the mechanisms of activation and

recruitment of monocytes to compose the activated microglia in the hypothalamus of experimental mice fed on high-fat diet (HFD), and the role played by TLR4 in this process.
Materials and methods: TLR4 defective mutant and wild-type (WT) mice were irradiated in a cobalt 60 source and submitted to BM transplantation from C57Bl6/GFP (with green fluorescence cells-GFP) or wild-type donors.To evaluate possible differences in activation/migration of microglial cells in the presence or absence of functional TLR 4, irradiated WT mice received BM from mutants ormutant mice were irradiated and submitted to BM transplant from mutants again. After recovery, mice were fed on a HFD for 8 weeks. After transplantation, GFP was detected by immunofluorescence co-stanning with macrophage markers in hypothalamic sections. RT-PCR was performed to evaluate the expression of genes involved in inflammation and ELISA was used to detect fracktalkine in the serum.
Results: Upon HFD feeding, active microglia cells are present in the hypothalamus after 8 weeks. When TLR4 mutant mice received BM from WT donors, microglial cells harboring WT-TLR4 are detected in the hypothalamus after 8 weeks; and, differently of TLR4 mutant mice, which are protected from diet-induced obesity (DIO), these transplanted mice present increased body mass, similar to WT mice, fed on HFD.
Conclusions: Therefore, the expression of a functional TLR4 is required to warrant the increase of adiposity during high-fat feeding. Amongst a number of chemokines evaluated, fractalkine was the one presenting highest and earliest levels in the hypothalamus of DIO mice. Thus, hypothalamic microglia cells originate from periphery by, at least in part, the action of fractalkine released from neurons.

P0350
Scavenger receptors SR-A/CD204 and CD36 differ markedly in signaling abilities but both receptors inhibit Th1 polarization of immune response

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Purpose/Objective: Scavenger receptors SR-A/CD204 and CD36 are two major receptors responsible for oxidized low density lipoprotein (LDL) uptake by macrophages, leading to the transformation of these cells into foam cells in atherosclerotic plaques. Both receptors also function as pattern recognition receptors for bacteria and other pathogens, but studies on their roles have been hampered by the lack of selective ligands.
Materials and methods: We studied effects of specific SR-A or CD36 ligation with antibodies on nitric oxide and cytokine production by peritoneal macrophages (PEMs) as well as on functions of these cells in antigen presentation.
Results: Our results indicate that, upon ligation by specific antibody or by dextran sulfate, SR-A mediates inhibition of lipopolysaccharide (LPS)-stimulated production of interleukin (IL)-6 and of p40 subunit of IL-12, enhances production of IL-10, but has no effect on tumor necrosis factor-α, nitric oxide or chemokine RANTES production. Surprisingly, acetylated LDL behaved as an antagonist of SR-A. In contrast to anti-SR-A antibody, anti-CD36 antibody alone stimulated production of all inflammatory mediators tested in macrophages, with IL-10 production being exceptionally high. Results of further experiments revealed that effects of anti-CD36 antibody were mediated by Toll-like receptor 2 (TLR2), but not by TLR4, whereas effects of SR-A ligation were mediated by heterotrimeric Gi/o proteins and by phosphatidylinositol 3-kinases. In an *in vitro* system of antigen presentation by macrophages to specific $CD4^+$ lymphocytes both anti-CD36 and anti-SR-A mAbs inhibited production of the Th1-type cytokine interferon-γ.
Conclusions: Our results have revealed that, despite binding overlapping sets of ligands and being involved in the same macrophage functions, SR-A and CD36 differ markedly in signaling abilities. Selective effects of SR-A ligation contrast with generalized enhancement of immune responses occurring in SR-A-deficient macrophages, which seems to be largely unrelated to the loss of the receptor function of SR-A.

P0351
Serum albumin modulates pro-inflammatory responses induced by bacterial lipopolysaccharides in macrophage-like MM6 cells

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Purpose/Objective: Bacterial lipopolysaccharide endotoxins (LPS) trigger potent and potentially life-threatening inflammatory responses. LPS-binding serum factors (LBP and CD14) and cell surface receptors (including CD14, CD55 and TLR4-MD2) of macrophages are key facilitators of these responses. Human serum albumin (HSA), the most abundant serum protein, is well characterised as a fatty acid carrier and regulator of blood pH. In addition, HSA has been implicated in host responses to invading pathogens and LPS. However, the role of HSA therein remains largely uncharted. Here, we sought to understand the molecular mechanisms associated with HSA-LPS interactions and their impact on host macrophage responses to LPS.
Materials and methods: We applied a combination of proteomic ligand-binding assays (surface plasmon resonance, ITC and PAGE-based mobility shift assays), to elucidate the molecular mechanisms associated with HSA-LPS interactions, and immunological techniques (ELISA and flow cytometry), to assess the impact of HSA-LPS interactions on the activation states of macrophage-like Mono Mac 6 cells.
Results: We found that the O-Antigen of LPS weakens the HSA-LPS interaction whereas truncations in the core region of LPS strengthen HSA binding. The interaction between HSA and LPS led to disaggregation of LPS multimers and the resultant HSA-LPS complexes target CD55 and LBP but not CD14 and TLR4-MD2. Activation of monomac 6 human macrophage-like cells, in terms of proinflammatory cytokine secretion and surface expression of LPS-related receptors, by different LPS chemotypes was markedly lower in the presence of HSA.
Conclusions: Our data suggests that HSA reduces the toxicity of LPS and thereby plays a pivotal role in mitigating the innate phase host response to invasive gram negative infection.

P0352
STING-dependent type I IFN induction by a cell permeable small molecule

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Purpose/Objective: The innate immune system uses germline-encoded pattern recognition receptors to sense invading pathogens. The detection of conserved microbial structures initiates antimicrobial defense mechanisms that are geared to initiate immediate effector functions and to induce adaptive immune responses. In this respect, cytokines of the type I interferon family (IFNs) play an important role. Therefore, a well-defined type I IFN inducer is desirable, as this would allow targeted clinical applications.
Materials and methods: We tested various small molecule compounds in murine bone marrow cells *in vitro* for their capacity to induce antiviral activity. In this regard we studied IRF3 phosphorylation, type I IFN induction at mRNA and protein level.

Results: We characterized the mode of action of a cell permeable small molecule that strongly triggers type I IFN through robust activation of the TBK1/IRF-3 route. Our data demonstrate that this small molecule signals towards type I IFN production in a TLR- and RLR-independent manner, as stimulation of MYD88-, TRIF- and MAVS-deficient cells still results in potent type I IFN responses. Indeed, we can show that the loss of the ER resident sensor molecule STING leads to a complete abrogation of cytokine production. Furthermore, *in vivo* experiments using this compound as a vaccine adjuvant suggest that targeting STING allows the induction of potent adaptive immune responses.
Conclusions: Altogether, these data for the first time establish a selective, cell-permeable activator of the STING pathway, opening new avenues for therapeutic applications.

P0353
Studies on the molecular mechanisms controlling the differential capacity of human neutrophils and monocytes to express IL-6 in response to LPS

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Purpose/Objective: IL-6 is a pleiotropic interleukin that acts either as pro-inflammatory or as anti-inflammatory cytokine. While human monocytes are well known to express IL-6 upon appropriate stimulation, whether human neutrophils do so remains still controversial. The aim of this study was to clarify, at molecular level, whether human neutrophils are able to express IL-6 in response to one of its major inducer in monocytes, namely lipopolysaccharide (LPS).
Materials and methods: Human neutrophils (>99.7% purity) and autologous Percoll-purified monocytes were isolated from buffy coats and incubated with 100 ng/ml ultrapure LPS for IL-6 gene expression studies by RT-qPCR, as well as for IL-6 release by ELISA (detection limit = 10 pg/ml). Under the same experimental conditions, both Polymerase II (Pol II) recruitment and histone 4 acetylation (H4Ac) at the IL-6 promoter were also assessed by chromatin immunoprecipitation (ChIP) assays.
Results: Neutrophils stimulated with LPS for 24 h released no or negligible amounts of IL-6 (<10 pg/ml), unlike autologous monocytes (> 10 ng/ml). In accordance, neither IL-6 mRNA, nor IL-6 primary transcripts (PT), could be detected in neutrophils stimulated with LPS for up to 6 h, contrary to what observed in monocytes. An absence of Pol II recruitment at the IL-6 promoter of LPS-stimulated neutrophils confirmed the lack of any IL-6 transcriptional activity. In contrast, significant levels of Pol II binding at the IL-6 promoter could be detected in LPS-treated monocytes as early as after 1 h of stimulation, and further increasing up to 5 h. Consistent with the latter findings, major differences were found between neutrophils and monocytes in terms of H4Ac levels (an epigenetic marker of transcriptionally active chromatin) present at the IL-6 locus. In fact, while H4Ac was readily detectable at the IL-6 promoter of resting monocytes, its levels dramatically increased after a 3 h LPS-stimulation. By contrast, neither constitutive, nor LPS-induced H4Ac recruitment at the IL-6 promoter was observed to occur in neutrophils.
Conclusions: Taken together, these results suggest that the differential capacity of human neutrophils and monocytes to express IL-6 upon LPS activation is likely controlled by epigenetic mechanisms acting at the promoter level.

P0354
The regulated IRAP-containing storage endosomes affect TLR9 signaling in dendritic cells

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Purpose/Objective: Nucleic acids sensing by Toll-Like Receptor 9 (TLR9) induces an innate immune response that subsequently can enhance the adaptive immune response. Dendritic cells (DCs) play a central role in both immune responses. While the innate immune response relays on intracellular TLR9 translocation from the endoplasmic reticulum (ER) into the endo-lysosomal pathway, the adaptive immune response depends on the antigen presentation ability of the DCs. Full activation of TLR9 requires: 1) cleavage in the endosomes and 2) an association with MyD88, which recruits in $VAMP3^+$ early endosomes NF-Kb, for inflammatory cytokines production, and in lysosomes IRF7 for type I IFN induction. Antigen cross-presentation requires the antigen processing machinery, which in DCs includes the antigen trimming aminopeptidase Insulin Responsive AminoPeptidase (IRAP). The aim of this study is to investigate the possibility of a cross talk between the antigen cross-presentation endosomes ($IRAP^+$) and the TLR9 signaling endosomes.
Materials and methods: Using IRAP deficient and wt mice and DCs, we assess the inflammatory cytokines and type I IFN production after TLR9 stimulation, as well as TLR9 localization and it's trafficking after DCs stimulation by TLR ligands.
Results: The fluorescence microscopy studies demonstrated that the SNARE VAMP3 co-localize with IRAP and other markers in the regulated storage endosomes (Syntaxin 6 and Rab14). This co-localization has a functional impact since DCs and mice inactivated for IRAP show hypersensitivity to TLR9 ligands, as illustrated by an increase in the synthesis and secretion of pro-inflammatory cytokines and type-I IFNs in comparaison with wt.
Conclusions: In conclusion, the $VAMP3^+$ endosomes are identical to cell specific regulated storage endosomes that contain the antigen trimming aminopeptidase IRAP and TLR9. This implies that in the absence of IRAP, TLR9 trafficking is altered and consequently signaling is enhanced.

P0355
The regulation of Interleukin-10 and Interleukin-12 production in macrophages: Investigating the differential production of IL-10 and IL-12 in C57BL/6 and BALB/c mice

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Purpose/Objective: The regulation of the immune response to pathogens is important to ensure their successful clearance with minimal host damage. Pattern recognition receptors (PRR) detect microbial products and induce cytokines such as interleukin-10 (IL-10) and interleukin-12 (IL-12) which shape the immunological response. IL-12 is a pro-inflammatory cytokine important for the differentiation of T helper 1 (Th1) cells which are critical for protection against intracellular pathogens. IL-10 is an immunosuppressive cytokine that amongst other functions can regulate the innate immune system by dampening dendritic cell and macrophage activity. IL-10 consequently minimises immune-driven host pathology during infection, but can also result in defective pathogen clearance. The regulation

of IL-10 and IL-12 is therefore of interest due to the central roles of these cytokines in the orchestration of an effective but regulated immune response.

Materials and methods: We have analysed PRR induced IL-10 and IL-12 production in macrophages at the protein level by ELISA and at the transcriptional level by qPCR. Microarray analysis and an investigation into the activation of various signaling pathways downstream of PRR stimulation by western blotting have also been carried out to further elucidate factors regulating the innate production of IL-10 and IL-12.

Results: We compared PRR driven cytokine responses of macrophages derived from various inbred strains of mice. We observed that macrophages generated from C57BL/6 and BALB/c mice, two commonly used strains which differ significantly in their resistance to several pathogens, produced reciprocal levels of IL-10 and IL-12 in response to several purified TLR ligands and heat-killed Gram-negative bacteria. Using this C57BL/6 versus BALB/c comparison as a model for our studies, we were able to further dissect the mechanisms regulating IL-10 and IL-12 and identified a central role for autocrine factors in the regulation of innate cytokine production.

Conclusions: Our investigation has revealed a mechanism for differential cytokine production observed in PRR activated C57BL/6 and BALB/c macrophages. These findings highlight key pathways responsible for the regulation of IL-10 and IL-12 and may provide valuable information on underlying factors contributing to the ability of these mouse strains to clear bacterial infections.

P0356
The role of 2′O-methylated tRNA in bacterial infections

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Purpose/Objective: RNA is a potent immune stimulator for innate immune receptors such as Toll-like receptors (TLR). We analysed the immunostimulatory potential of bacterial transfer RNA (tRNA) from different strains in human PBMCs and FLt3l- derived murine pDCs *in vitro*.

Since 2′O-methylations in RNA molecules have been shown to act inhibitory on IFN-α production, we further focused on 2′-*O*-methylguanosin at position 18 (Gm18) of the tRNA molecule, which is introduced by the (Gm18)-2′O-ribose-methyltransferase trmH.

Materials and methods: Stimulation experiments of human PBMCs and murine FLt3l-derived DCs were conducted with tRNA of different bacterial strains. The bacterial tRNA was isolated by anionic exchange chromatography. For stimulation experiments tRNA was complexed to the cationic lipid DOTAP. IFN-α secretion was determined via ELISA.

We generated an *E. coli-Nissle* trmH-knock-out strain (EcN*Δ*trmH) by using the red-recombinase system.

Results: We could show that bacterial tRNA induces IFN-α production in human PBMCs and murine FLt3l-induced DCs via TLR7. Surprisingly it turned out, that a single 2′O-methylation of a conserved guanosine at position 18 (Gm18) of the tRNA molecule in some bacterial strains abrogated the IFN-α secretion. E. coli-Nissle wildtype tRNA did not trigger IFN-α secretion whereas tRNA derived from EcNΔtrmH strain leads to IFN-α production in murine pDCs.

Additionally the Gm18-containing tRNA from *E. coli-Nissle* showed an inhibitory effect on TLR7-activation and acts as a receptor antagonist.

Conclusions: Our results demonstrate that the IFN-α secretion may be regulated by the activity of the enzyme trmH and the amount of Gm18 methylation. Thus, the control of an IFN-α response in the gut might be beneficial for the pathogenity of bacteria and a tightly regulated IFN-α response may be helpful for the treatment of inflammatory bowel diseases.

One approach for answering these questions could be the recolonization of germ-free mice with EcNΔtrmH and different bacterial strains with subsequent DSS-colitis model. If any differences in the extent of the inflammatory response can be observed, one could gain new insights of the importance of the gut flora in connection with inflammatory bowel diseases.

P0357
The role of dsRNA receptors in human renal tubular epithelial cells during infection with BK virus

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Purpose/Objective: The polyomavirus BK (BKV) frequently reactivates in kidney transplant recipients leading in about 5% of the patients to BKV-associated nephropathy, at present an important cause for graft dysfunction. Impaired immune surveillance, lytic viral replication in renal tubular epithelial cells (TECs), and allo-immune activity are factors that seem to contribute to the development of nephropathy. We previously found that the viral dsRNA receptors Toll-like receptor 3 (TLR3), melanoma differentiation associated protein 5 (MDA5) and retinoic acid inducible gene-I (RIG-I) regulate anti-viral, pro-inflammatory and pro-apoptotic responses in primary TECs. Here, we aim to study the interaction between TECs and BKV and address the involvement of dsRNA receptors in the immune response against the virus.

Materials and methods: dsRNA receptor expression was analysed by quantitative PCR and immunohistochemistry in renal transplant biopsies obtained during BKV infection ($n = 7$) and stable graft biopsies ($n = 5$) and in primary TECs infected *in vitro* with a clinical isolate of BKV. Expression of both viral genes and dsRNA sensors was determined by immunofluorescence and PCR.

Results: TLR3, MDA5 and RIG-I transcription was significantly enhanced in BKV infected biopsies ($P < 0.01$), a feature of dsRNA receptor activation. Immunohistochemistry demonstrated that tubuli expressed all three sensors. In primary TECs, BKV infection was characterised by expression of the viral protein 1 and large T antigen, and nuclear accumulation of viral proteins. Cytopathic changes became apparent 1 week after infection leading to cell lysis and release of infectious particles. At 6 and 48 h after BKV infection, dsRNA receptor expression was only marginally enhanced. These findings suggest that either BKV does not generate dsRNA molecules that are recognized by TLR3, MDA5 or RIG-I or that the virus suppresses antiviral immune responses and/or the production of type I interferons in primary TECs.

Conclusions: BKV infection is associated with increased expression of dsRNA receptors in renal transplant biopsies, yet expression of TLR3, MDA5 and RIG-I was hardly enhanced in BKV infected primary TECs.

P0358

The role of soluble Toll-like receptor 3 in human astrocytes

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Purpose/Objective: Toll-like receptors (TLRs) are a group of pattern-recognition receptors that plays an important role in innate immune response against bacterial and viral infections. Among them, intracellular TLRs are localized in endolysosomal compartment and recognize foreign nucleic acids from a variety of pathogens. Recent studies have shown that soluble form of Toll-like receptor 9 (sTLR9) generated by proteolytic process is negatively regulates TLR9 signaling. Similarly, we found that a soluble Toll-like receptor 3 (TLR3) is generated by alternative splicing in human astrocytes. In this study, we examined whether a soluble TLR3 has negatively effect on TLR3 signaling in human astrocytes.
Materials and methods: Human astrocytes were transfected with a mock vector or a plasmid encoding soluble TLR3 and treated with poly(I:C) for different times. The lysates were analyzed by western blot using anti-phospho-IRF3, anti-IRF3, anti-phospho-IκBα, anti-IκBα, anti-TLR3 as well as anti-tubulin antibodies. And human astrocytes were co-transfected with a plasmid encoding TLR3-CFP and sTLR3-EGFP and treated LysoTracker either with or without poly(I:C) stimulation for 1 h and cells were fixed and analyzed by confocal microscopy. Also human astrocytes were incubated with poly(I:C)-coated beads and lysates were analyzed by western blot using anti-TLR3 antibody.
Results: poly(I:C)-induced the activation of IRF3 and IκBα was decreased in human astrocytes that express soluble TLR3 compared to control cells. And soluble TLR3 is co-localized with wild type of TLR3 in lysosomal compartment when poly(I:C) was treated. Intriguingly, soluble TLR3 binds poly(I:C) as wild type TLR3 does in human astrocytes.
Conclusions: Our results suggest that soluble TLR3 generated by alternative splicing negatively regulates TLR3 signaling through binding ligands [ex. poly(I:C)] competitively with wild type TLR3.

P0359

The role of UNC93B1 in localization of TLR3 on plasma membrane and in cell priming to nucleic acid agonists

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Purpose/Objective: Appropriate localization of nucleic acid-sensing TLRs (NAS TLRs) prevents autoimmune response. We observed that upregulation of hUNC93B1 promoted localization of differentially glycosylated hTLR3, but not other NAS TLRs at the plasma membrane. We set to investigate determinants of trafficking hTLR3 towards the plasma membrane and physiological processes that regulate transcription of *hUNC93B1*.
Materials and methods: We prepared chimeric constructs, where transmembrane segments or cytosolic domains of hTLR3 and hTLR9 have been exchanged. Their localization was visualised with confocal microscope.

Human umbilical vein endothelial cells (HUVEC) were stimulated with several TLR ligands or IFN-β. mRNAs of hUNC93B1and hTLR receptors were measured with qPCR. Differentially glycosylated form of hTLR3 was detected with WB. Surface localization of hTLR3 was detected with flow cytometer.

Human *UNC93B1* promoter region was examined for transcription factor binding sites using the MatInspector software.

For priming experiment Ramos-Blue cells were pretreated with the first agonist and subsequently treated with another agonist. Agonists used were poly(I:C) and CpG ODN. Activation of NF-κB/AP-1-inducible reporter was measured with a spectrophotometer.
Results: We determined that hTLR3 surface sorting motif for the UNC93B1-dependent surface localization resides in the hTLR3 ecto-domain. The analysis of *hUNC93B1* gene promoter region revealed that its transcription is regulated by several transcription factors triggered by the TLR3 activation and type I interferon signalling. Poly(I:C) and IFN-β upregulated transcription of UNC93B1 and increased the amount of differentially glycosylated hTLR3 at the surface of HUVEC cells. We demonstrated the physiological relevance of a positive feedback loop based on the upregulation of hUNC93B1 after hTLR3 stimulation by selectively sensitizing the responsiveness to hTLR9 stimulation after the prior exposure to poly(I:C), but not to ODN.
Conclusions: Our findings identified hTLR3 as the master regulator of hUNC93B1 that in turn governs the responsiveness of all NAS TLRs. The low amount of endogenous TLR3 agonist and the corresponding low risk of autoimmune reaction make this receptor an appropriate sentinel of viral infection at the cell surface and a regulator for sensitizing the response of NAS TLRs through upregulation of UNC93B1.

P0360

TLR4 expression in bone marrow-derived cells modulate diet-induced inflammation in insulin sensitive tissues

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Purpose/Objective: Recent studies have shown that saturated fatty acids from high-fat diets induce the activation of a TLR4-dependent innate immune response in insulin-sensitive tissues leading to insulin resistance and glucose intolerance. Mice lacking a functional TLR4 (C3H/HeJ) are protected from diet-induced obesity and show an improved response to insulin. The TLR4 receptor is expressed predominantly in immune cells, however a number of other tissues also can express this receptor. It is currently unknown if the TLR4-dependent impairment of insulin signaling is due to the activation of TLR4 receptors in immune cells and/or in insulin-sensitive tissues. The objective of the present study was to investigate if a defective TLR4 signaling only in bone-marrow derived cells is sufficient to modulate the whole-body insulin action.
Materials and methods: C3H/HeJ and wild-type (WT) mice were irradiated in a cobalt 60 source and submitted to bone marrow transplantation (BMT). After recovery, mice were fed on a high fat diet (HFD) or standard rodent chow for 8 weeks. Inflammatory markers were evaluated in the liver and visceral adipose tissue. In addition, we evaluated food intake, body mass gain, energy expenditure and the activation of markers of thermogenesis in brown adipose tissue. WT transplanted with BM from TLR4 mutant mice are protected from body mass gain in HFD.
Results: When TLR4 mutant mice received BM from WT donors this effect was blunted and mice gained weight. TLR4 mutant mice presented an increase in energy expenditure in chow when compared to WT mice, which was accompanied by and increased UCP1 mRNA expression in brown adipose tissue. HFD TLR4 mutant mice have an increased IL-10 mRNA expression both in liver and adipose tissue,

which was not observed to WT mice. This IL-10 increase also occurred in WT transplanted with TLR4 mutant BM and was blunted in TLR4 mutant transplanted with WT BM. TLR4 mutant mice fed on HFD also presented reduced IL-6 mRNA levels in liver, which was not observed in WT.

Conclusions: Thus, we show that TLR4 mutant mice are protected from diet-induced activation of inflammation in the liver, and present increased energy expenditure, suggesting that TLR4 signaling play a role in the control of whole body energy homeostasis.

P0361

TLR4 signalling shapes actin cytoskeleton to modulate B cell migration

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Purpose/Objective: Naive B cells are constantly migrating throughout the follicular dendritic cell network in the follicles of secondary lymphoid organs. This motility is promoted in part by the chemokine CXCL13 and allows B cells to scan for potential antigens. Specific antigen recognition by the B cell receptor (BCR) triggers the humoral immune response. In addition, pathogenic molecules that reach the follicles can be recognized by receptors other than BCR as the Toll-like receptor (TLR) family. They recognize conserved molecular patterns from bacteria and virus, and triggers innate immune responses. Little is known regarding the effects of innate signals on B cell behaviour and the possible effects of this modulation in B cell function.

Materials and methods: We combined time-lapse microscopy and a 2D model of haptokinesis.

Results: Here we show that short-time stimulation via TLR4 enhances the B cell response to CXCL13 by increasing their polarization, adhesiveness and motility. This seems to be dependent on the MyD88-mediated signalling cascade of TLR4. The B cell chemotactic response to CXCL13 in Boyden chambers is also enhanced by TLR4 stimulation; this is accompanied by a twofold increase in the expression levels for CXCL13 receptor, CXCR5, at the B cell surface. We observed that TLR4 stimulated B cells have higher F-actin content and higher basal and CXCL13-triggered activation of the small Rho-GTPase Rac than non-stimulated B cells; no detectable differences were observed for the small Rho-GTPase Cdc42.

Conclusions: Thus, short-time stimulation through TLR4 seems to prime the actin cytoskeleton of naïve B cells for subsequent CXCL13 responses; this may improve the B cell chances to encounter a cognate antigen.

P0362

Toll-like receptor 1 and 2 play a role in recognition of *Coxiella burnetii* in humans

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Purpose/Objective: The bacterium *Coxiella burnetii* is the causative agent of Q fever. Acute Q fever presents with fever, pneumonia or hepatitis, whereas chronic Q fever develops over several months to years following infection and is characterized by endocarditis or endovascular infection.

Toll-like receptors comprise a family of receptors which recognize molecular structures that are conserved in many microbial products. TLRs play a major role in activating the innate immune system. In this study, we investigated the role of specific TLRs in the recognition of *C. burnetii* in humans.

Materials and methods: Peripheral blood mononuclear cells were isolated from healthy donors. TLR function was inhibited using specific antibodies of either TLR1, TLR2, TLR4, TLR6 or TLR9. After 60 min of preincubation, PBMCs were stimulated with the heat killed reference strain *C. burnetii* Nine Mile. After 24, 48 h, or 7 days of stimulation, cytokine responses in the supernatant were measured. Furthermore, we studied the influence of functional single nucleotide polymorphisms (SNPs) in the different TLR genes regarding the recognition of *C. burnetii*. Therefore PBMCs of individuals, who were completely genotyped for polymorphisms in TLR1 (R80T, N248S, andS602I), TLR2 (R753Q and P631H), TLR4 (D299G and T399I), or TLR6 (S249P) were stimulated with the Dutch outbreak isolate *C. burnetii* X09003262. ELISA was used for cytokine measurements.

Results: Stimulation of PBMCs with *C. burnetii* Nine Mile resulted in a robust IL-1β, TNF-α, IL-6, IL-10, IFN-γ, IL-22 and IL-17 induction. The induction of proinflammatory cytokines, such as IL-1β, TNF-α and IL-6, was not affected after inhibition of TLR1, TLR4, TLR6 or TLR9. However, inhibition of TLR2 resulted in decreased production of IL-1β and IL-6. The SNP analysis confirmed the role of TLR2 regarding the recognition of *C. burnetii*, as individualsbearing the TLR2 R753Q SNP showed higher IL-1β and TNF-α induction. The anti-inflammatory cytokine IL-10 was not affected after inhibition of either TLR1, TLR4 or TLR6. In contrast, IL-10 induction decreased after TLR2 inhibition. Surprisingly, individuals homozygous for all three SNPs in TLR1 showed less production of IL-10.

Conclusions: These results indicate a role for TLR1 and TLR2 in recognition of *C. burnetii* and the induction of an early immune response in humans.

P0363

Tumor progression and resistance to chemotherapy induced by TLR7 stimulation in non small cell lung carcinoma

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Purpose/Objective: Novel cancer immunotherapies using TLR7 agonists are being developed, which are based on the amplification of immune responses. However, our recent studies demonstrate a pro-tumoral effect and resistance to chemotherapy induced by TLR7 stimulation of human lung tumor cell lines *in vitro*. Studies performed on human tumoral tissues revealed that TLR7 is both expressed *in situ* by tumor cells and by immune cells infiltrating tumor stroma (Cherfils-Vicini J. et al. JCI. 2010). Indeed *in vivo* TLR7 stimulation by its agonists, can lead to tumor progression or to tumor regression, through activation of antitumoral immune cells.

Materials and methods: In the present study human A549 NSCLC cells were grafted onto immunodeficient NOD/SCID mice, and murine LL/2 lung squamous cell carcinoma cells were grafted onto either NOD/SCID or immunocompetent C57Bl/6 mice.Tumor growth was followed after injection of TLR7 agonists and/or chemotherapy.

Results: We observed that injection of the TLR7 agonist, loxoribine, in NOD/SCID mice induces increased tumor growth and resistance to cisplatin of A549 cells compared to unstimulated conditions.These

results confirm the chemoresistance observed *in vitro*. On the contrary, loxoribine had no effect on LL/2 cells *in vitro* or *in vivo*, when grafted in NOD/SCID mice and had an anti-tumoral effect when LL/2 cells were grafted on C57Bl/6 mice. Interestingly an additional TLR7 agonist, CL264, induced tumor progression and resistance to chemotherapy when LL/2 cells were grafted in NOD/SCID mice. In immunocompetent C57Bl/6 mice low doses of CL264 induced anti-tumoral effects whereas high doses of CL264 induced pro-tumoral effects and chemo-resistance.

Conclusions: Taken together, these results indicate a fine regulation of TLR7 stimulation on both the immune system and tumoral cells, depending on the dose of the TLR7 ligand. Cancer immunotherapies using TLR7 agonists should take into account these new observations.

P0364

Virus-cell fusion as a trigger of innate immunity dependent on the adaptor STING

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Purpose/Objective: The innate immune system senses infection by detecting either evolutionarily conserved molecules essential for the survival of microbes or abnormal location of molecules.Recent research on the recognition of virus by innate immune mechanisms has been focused on the detection of nucleic acids derived either directly from the incoming virus or from RNA or DNA intermediates produced during viral replication. In this project, we investigated a novel mechanism for detection of infection with enveloped virus, which is independent of DNA or RNA detection.

Materials and methods: Virus-cell fusion was studied using virus like particles (VLPs) from HSV-1, which contain no genomic material or viral capsid but retain the ability to fuse with target cells. We used these VLPs to stimulate cells from mice deficient in signaling pathways previously shown to participate in innate recognition of virus. To study the fusion event further we also used two distinct cell-cell fusion assays based on the HIV fusion protein Env and highly fusogenic liposomes.

Results: We demonstrate the existence of a previously unknown innate detection mechanism induced by fusion between viral envelopes and target cells. Virus-cell fusion specifically stimulated a type I interferon response with expression of interferon-stimulated genes, *in vivo* recruitment of leukocytes and potentiation of signaling via Toll-like receptor 7 (TLR7) and TLR9. The fusion-dependent response was dependent on the stimulator of interferon genes STING but was independent of DNA, RNA and viral capsid.

Conclusions: We suggest that membrane fusion is sensed as a danger signal with potential implications for defense against enveloped viruses and various conditions of giant-cell formation.

Poster Session: Sensing and Communicating – Lectins and Lectin-Like Receptors

P0366
Characterisation of the myeloid inhibitory C-type lectin-like receptor (mMICL) knock-out mouse

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Purpose/Objective: The myeloid inhibitory C-type lectin-like receptor (mMICL) is expressed on cells of myeloid lineage including neutrophils.The presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail and the widespread expression of its endogenous ligand/s in various tissues may suggest a role for this receptor in the maintenance of immune homeostasis and/or the regulation of inflammatory responses. To address these possibilities, we describe the generation and characterisation of the mMICL knock-out mouse.
Materials and methods: mMICL expression in wild-type and knock-out mice under steady state conditions was determined in tissues including peripheral blood, spleen and bone marrow. The distribution and composition of different myeloid and lymphoid populations in these tissues was also compared. mMICL knock-out and wild-type mice were aged for 24 months in order to assess any spontaneous development of disease. Furthermore, animals were also subjected to various models of sterile inflammation.
Results: In wild-type animals, mMICL showed high levels of expression in all immune tissues and was particularly pronounced in the bone marrow. No differences were observed in the distribution or composition of any myeloid or lymphoid cell populations, in the knockout mice. Ageing mMICL knock-out mice also showed no signs of spontaneous disease development.In a model of thioglycollate-induced peritonitis, mMICL expression in the recruited peritoneal leukocytes of wild-type mice reached a peak at 4 h which then decreased at 24 h and at later time points.Under the same conditions, mMICL knock-out mice showed a significantly greater recruitment of neutrophils 4- and 24-h post-i.p.
Conclusions: Our data suggests that, under steady state conditions, the absence of this inhibitory receptor does not contribute to the disruption of immune homeostasis. However, in response to inflammatory stimuli, early neutrophil-mediated responses are exacerbated. The mechanisms by which mMICL negatively regulates these responses are currently being investigated.

P0367
Characterisation of the orphan gene CLEC2L

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Purpose/Objective: Natural Killer Gene Complex (NKC) encoded C-type lectin-like receptors (CTLRs) are expressed on various immune cells including T cells, NK cells and myeloid cells and thereby contribute to the orchestration and modulation of cellular immune responses. Certain members of these NKC-encoded CTLRs are grouped into the C-type lectin domain family 2 (CLEC2 family) that interact with CTLRs of the NKRP1 family expressed on cytolytic lymphocytes, e.g. AICL (encoded by *CLEC2B*) with NKp80. Noteworthily, CLEC2 and NKRP1 receptors are tightly genetically linked. While most CLEC2 family members are expressed by hematopoietic cells (e.g. CD69, AICL), we recently reported skin-restricted expression of the CLEC2 family member KACL (*CLEC2A)* on keratinocytes. Here we provide the first characterization of *CLEC2L*, another orphan gene of the CLEC2 family.
Materials and methods: *CLEC2L* tissue expression was addressed by quantitative real-time PCR. *CLEC2L* mutants were generated by site-directed mutagenesis. *CLEC2L*-encoded proteins and mutants were ectopically expressed in 293T cells for characterization by flow cytometry and immunoblotting. Soluble CLEC2L ectodomains were expressed in 293T cells and used for the generation of CLEC2L-specific antisera in chicken. Endogenous CLEC2L expression was addressed by immunohistochemistry using CLEC2L antisera.
Results: In contrast to other CLEC2 family members, the coding sequence of *CLECL* is highly conserved among mammals and the *CLECL* locus located outside of the NKC. Interestingly, expression of *CLEC2L* is also highly tissue-specific: *CLEC2L* transcripts are abundant in brains of man and mice, but present only at low levels or barely detectable in other tissues. Hence, we termed the *CLECL* gene product BACL (brain-associated C-type lectin). We show that BACL is readily expressed on the cell surface as homodimer linked by two disulfide bonds in the stalk region. BACL-specific antisera revealed strong expression of BACL by Purkinje neurons.
Conclusions: Here, we provide the first characterization of the orphan gene *CLEC2L* encoding a highly conserved, non-NKC-encoded, disulfide-linked, homodimeric CTLR of the CLEC2 family predominantly expressed in the brain, and thus termed BACL.

P0368
Galectin-1 secretion by mature dendritic cells supress *in vitro* Th-17 differentiation in humans

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Purpose/Objective: To determine the role played by galectin-1 secreted by mature dendritic cells (mDCs) in the differentiation of T helper cells, focusing on the generation of Th17 cells and the secretion of IL-17 *in vitro*.
Materials and methods: Human inmature dendritic cells were obtained by isolation and differentiation of peripheral blood monocytes using GM-CSF and IL-4. Immature dendritic cells were incubated with IL-1b, TNFa, IL-6 and prostaglandin E2 to obtain mDCs. To investigate the role of mDCs-secreted galectin-1 a mixed lymphocyte reaction using purified T cells (1:1 ratio T : mDC) was set up with or without a neutralising antibody to galectin-1. After the stimulation period, cytokine synthesis by T cells was analysed by ELISPOT and both galectin-1 and cytokines in the culture media by ELISA.
Results: Our results shows that mDCs synthesise and secrete up to 47 times higher levels of galectin-1 in comparison to those secreted by naïve CD4 T cells polarised *in vitro* towards a Th1, Th2 or Th17 phenotype. When T cells were incubated stimulated with allogeneic mDCs in the presence of sufficient amounts of a neutralising antibody to galectin-1 it was found that the number of cells secreting IFN-g rise dramatically indicating that galectin-1 reduces Th1 cell differentiation *in vitro*, in accordance with previous data. In addition and for the first time we show that in humans, neutralisation of galectin-1 secreted by mDCs also promotes IL-17A secretion and Th17 cell differentiation *in vitro*.
Conclusions: In conclusion, human Th17 cell differentiation is regulated by mDC-secreted galectin-1 *in vitro*. These results could explain the T cell phenotype observed in some human diseases where the synthesis of galectin-1 is reduced such as type 1 diabetes.

P0369

Galectin-3 expression in Th cell subsets: reduced secretion of this immunomodulatory lectin in type 1 diabetes patients

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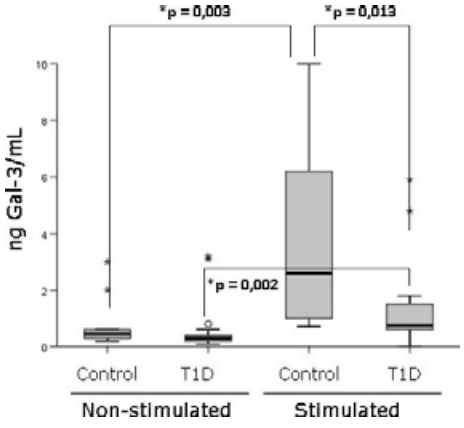
Purpose/Objective: To determine the expression and secretion of the immunomodulatory lectin galectin-3 in several T helper cell subsets (Th1, Th2 and Th17) and evaluate potential differences in the synthesis of this immunomodulatory lectin between healthy controls and Type 1 Diabetes (T1D) patients.
Materials and methods: naïve CD4 T cells from healthy donors were polarized under Th1, Th2 and/or Th17 conditions, and Tbet, GATA-3, RORC2 and galectin-3 expression was determined by real-time PCR. Intracellular galectin-3 was measured by western blot. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy controls and T1D patients. Secreted galectin-3 and cytokines were measured by ELISA.
Results: The *in vitro* polarization of naïve CD4 T cells towards a Th17 phenotype drives to the synthesis of elevated levels of galectin-3 in comparison with Th1 and Th2 subsets. In the case of T1D, CD3/CD28 stimulated PBMCs from these patients secrete significantly lower levels of galectin-3 when compared to those from healthy volunteers. This reduced secretion takes place in a pro-inflamatory environment, which is shown by an increased IFN-γ/IL-10 ratio.



Conclusions: Among the Th cell subsets, Th17 cells express the highest levels of galectin-3, suggesting an important role of the lectin in these cells. When this lectin is analyzed in the context of an autoimmune disease, such as T1D, diminished secretion of galectin-3 by PBMCs from those patients is observed; due to the apoptotic function of galectin-3 on T cells this defect could drive to the pathological survival of activated T cells, exacerbating the immune response.

P0370

Key role of the EGF receptor pathway in stress-induced upregulation of NKG2D ligands expression

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Purpose/Objective: The human MICA/B and ULBP(1−4) genes encode ligands for the activating receptor NKG2D expressed by several T cell subsets and NK cells. Of note, their surface expression is upregulated by several pathogens and by sterile stresses such as UV irradiation, oxidative stress, and transformation. This renders cells 'immunologically visible', reflecting which NKG2D and its ligands are common targets of immunoevasion. However, key mechanisms regulating NKG2D-ligand (NKG2DL) expression remain ill-defined. Upregulation by UVB has mostly been attributed to transcriptional activation induced by the DNA Damage Repair pathway. However, whereas surface NKG2DL expression is not detected, low levels of NKG2DL RNA are often detectable in healthy tissues, strongly suggesting post-transcriptional control mechanisms. As UV irradiation and other stresses can transactivate the EGF Receptor (EGFR), we investigated this as a pathway regulating NKG2DL RNA stability.
Materials and methods: Inhibition of EGFR greatly reduced upregulation of MICA/B and ULBP2 provoked by UVB and by other sterile stresses, consistent with which EGF upregulated cell surface NKG2DL expression and rendered cells NKG2D-dependent targets of cytotoxic NK and gd T cells. Canonical A/U rich elements (ARE) were identified in the 3' untranslated region of NKG2DL mRNAs which required re-annotation.
Results: The elements were distinct from microRNA binding sites previously identified in MICB RNA, and their profound destabilisation of mRNA was confirmed by Luciferase and GFP reporter systems. The RNA-binding proteins AUF1 and HuR were identified as targeting MICA/B and ULBP2 mRNAs for degradation. Finally, the signalling pathways (e.g. MAPKs) linking the EGFR to increased NKG2DL expression were also explored.
Conclusions: Our results provide new insight into stress-induced NKG2DL expression with a central role of the EGF receptor integrating responses to various stimuli long known to upregulate MICA/B and ULBPs. Of note, the EGFR pathway is often dysregulated in cancers, and may be integral to cell activation by viruses such as CMV.

P0371

Knockout of sialoadhesin enhances microglial accumulation during prion pathogenesis

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Purpose/Objective: The adhesion molecule sialoadhesin (Sialic acid-binding Ig-like lectin 1 [Siglec-1], CD169) binds sialic acid residues of glycoproteins and promotes endocytosis. Sialoadhesin is constitutively expressedby splenic marginal zone or lymph node subcapsular sinus macrophages which function to trap immune complexes. Sialoadhesin expression may also be induced in myeloid lineage cells upon exposure to blood-borne cytokines. The prion glycoprotein is heavily sialylated and may act as a ligand for sialoadhesin. To determine if sialoadhesin played a role in the uptake and pathogenesis of prion infectious-agent, scrapie challenge studies were performed in sialoadhesin deficient (SnKO) transgenic mice.
Materials and methods: Groups of SnKO mice and C57Bl/6 control mice were inoculated intracerebrally or intraperitoneally with mouse adapted scrapie agent ME7. Mice were studied at 5, 10 and 15 weeks for peripheral prion pathogenesis following intraperitoneal inoculation.

Results: Following intraperitoneal infection SnKO mice revealed no significant differences to C57Bl/6 mice in all aspects studied, including assessment of immune function via antigen trapping assays and disease-associated PrP accumulation in lymphoid follicles. Following intracerebral infection alterations were observed in brain region-specific microglial responses resulting in elevated levels of disease-associated vacuolation in hippocampal CA1 region and enhanced microglial proliferation and accumulation into multi-cellular aggregates.
Conclusions: The removal of sialoadhesin expression did not affect the peripheral pathogenesis of prion disease, therefore sialoadhesin is not required for the sequestration of the prion infectious agent into lymphoid germinal centres. Following intracerebral infection sialoadhesin-deficient mice displayed enhanced microglial response and proliferation in specific brain regions. These data suggest that the knockout of sialoadhesin may have altered microglial cell-cell recognition resulting in microglial multicellular aggregates. Alternatively damage to the blood brain barrier following intracerebral inoculation may have altered the priming of sialoadhesin-deficient microglia, leading to an enhanced pro-inflammatory response during infection and an increase in microglial proliferation.

P0372

Role of the C-type lectin receptor DCIR in cerebral malaria development

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Purpose/Objective: Malaria is one of the main global causes of death from infectious diseases. The vast majority of severe malaria cases and deaths are caused by infection with *Plasmodium falciparum* which is often associated with severe pathology including anemia, metabolic acidosis, impaired blood circulation, and cerebral malaria (CM). The murine *P. berghei* ANKA (PbA) infection is an established model of cerebral malaria, and blood-stage infection leads to CM induction in 80−100% of PbA-infected mice. While a number of studies have addressed the role of adaptive immunity in CM development, the contribution of pattern recognition receptors to CM is still poorly understood. C-type lectin receptors (CLRs) are of particular interest since engagement of CLRs on the one hand may lead to the initiation of immune responses but on the other hand may also result in maintaining immune homeostasis. The objective of this study was to investigate the contribution of the CLR dendritic cell immunoreceptor (DCIR) to CM development.
Materials and methods: $DCIR^{-/-}$ mice and the respective C57BL/6 wild-type control mice were infected i.p. with PbA-infected erythrocytes. Parasitemia was determined in Giemsa-stained blood smears from tail blood and mice were monitored daily for symptoms of CM. Leukocyte sequestration in the brain and the expression of activation markers by immune cells were measured by flow cytometry as well as immunohistochemistry. Cytokine levels were assessed by cytometric bead array. Statistical analyses were performed with unpaired Student *t* test and the log rank test to compare survival curves.
Results: Infection of C57BL/6 wild-type mice with PbA led to CM in 80% of infected mice, whereas $DCIR^{-/-}$ mice were highly protected from CM (only 20% CM development). Parasite loads were similar in $DCIR^{-/-}$ and wild-type mice indicating that DCIR deficiency did not affect parasite replication. To address the mechanism by which DCIR deficiency mediated protection from CM, leukocyte sequestration in the brain and levels of pro-inflammatory cytokines in serum were measured. Indeed, T cell sequestration was reduced in brains of PbA-infected $DCIR^{-/-}$ mice and cytokine levels in serum were markedly modulated compared to C57BL/6 wild-type mice.
Conclusions: This study indicates that DCIR is involved in CM induction, thus highlights the importance of CLRs for the innate immune response during the course of malaria.

P0373

Siglec-7 expression decreases in freshly isolated peripheral blood mononuclear cells (PBMCs) under diabetogenic conditions

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Purpose/Objective: In both, type 1 and type 2 diabetes mellitus; cytokine and chemokine production within pancreatic islets is detrimental for the insulin producing b-cells. Also, infiltration of macrophages in the pancreatic islets is a hallmark of islet inflammation. Siglecs (Sialic-acid binding immunoglobulin like lectins) are cell surface receptors expressed on haematopoietic cells which participate in such immune responses. We investigated their expression in freshly isolated PBMCs under diabetogenic conditions. Our experiments revealed decreased expression of Siglec-7 along with the activation of monocytes under such conditions.
Materials and methods: PBMCs were isolated from buffy coats of six non-diabetic individuals followed by culture with elevated glucose (22.2 mM)/ 0.5 mM palmitate, or cytokine mixture (2 ng/ml IL-1β/ 1000 U/ml IFN-γ) or 100 ng/ml Lipopolysaccharide (LPS), for 12 h. The mRNA expression of Siglec-7; immune cell marker CD25; as well as cytokine IL-6 was analyzed by RT-PCR. Siglec-7 was assessed by flow cytometry.
Results: LPS, elevated glucose and palmitate, as well as cytokine mixture induced activation of PBMCs as seen by the induction of IL-6 mRNA (380-, 80- and 50-fold, respectively), as well as by increased CD25 expression (45-, 10- and 5-fold, respectively) as compared to the untreated control ($P < 0.05$). Simultaneous decrease was observed in Siglec-7 mRNA expression; 80% by cytokine mixture, and 50% by elevated glucose and palmitate, whereas LPS decreased Siglec-7 mRNA expression only during the acute 2h-exposure (60% reduction).

The glucose/palmitate-induced effect was confirmed by flow cytometric analysis of Siglec-7 cell surface expression showed 35% decrease in percentage of Siglec-7 positive cells as well as 25% decrease in the mean fluorescence intensity.
Conclusions: Our results show that Siglec-7 is decreased under diabetogenic conditions wherein the PBMCs are in an activated state, hinting towards the inhibitory role of Siglec-7 in the activation of these immune cells during the development and progression of diabetes mellitus.

P0374

The Mincle-activating adjuvant TDB induces MyD88-dependent, TLR-independent Th1 and Th17 responses through IL-1R signalling

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Purpose/Objective: Vaccination is the most successful strategy to prevent potentially life-threatening diseases. Protective immunity is often dependent on strong cellular immune responses rather than antibody production. However, the most used human adjuvant aluminium hydroxide is a weak inducer of Th1 responses. Trehalose-6, 6-dibehenate (TDB), the synthetic analogue of the mycobacterial cord

factor trehalose-6, 6-dimycolate is a potent adjuvant not only inducing a strong Th1 but also a pronounced Th17 immune response. We identified the C-type lectin Mincle as receptor for these glycolipids that triggers the FcRγ-Syk-Card9 pathway for APC activation and adjuvanticity. While APC activation *in vitro* was MyD88-independent and solely required Mincle, *in vivo* data revealed the adjuvant effect of TDB was strongly reduced in MyD88$^{-/-}$ mice.

Materials and methods: Thus, we dissected which MyD88-dependent pathways contribute to TDB-mediated adjuvanticity after immunisation with a tuberculosis subunit vaccine *in vivo*. In order to further characterize adjuvant-induced immune responses, we analysed recruitment kinetics of innate immune cells after intraperitoneal or subcutaneous injection into C57/Bl6, Mincle$^{-/-}$ and MyD88$^{-/-}$ mice and followed the vaccine's and APC's fate to the draining lymph nodes.

Results: We show here that development of antigen-specific Th1 and Th17 immune responses crucially depended on IL-1/IL-1 receptor mediated signals, whereas IL-18 and IL-33 were dispensable. Interestingly, ASC-deficient mice had impaired IL-17 but intact IFNγ responses, indicating partial independence of TDB adjuvanticity from inflammasome activation.

Neutrophils and inflammatory monocytes were the first cell types being recruited to the injection site in very high numbers upon immunisation. Neutrophil influx was equally dependent on recognition via Mincle and MyD88 signalling. However, depletion of neutrophils did not impair generation of Th1 and Th17 immune responses. Experiments interfering with recruitment of inflammatory monocytes are currently ongoing.

Conclusions: Taken together, the glycolipid adjuvant TDB relies on MyD88-dependent but TLR-independent pathways for efficient Th1/Th17 adjuvanticity. Pharmacologic and genetic abrogation of IL-1R signaling identified IL-1 as the major MyD88-dependent factor induced by TDB through Mincle-Card9 signaling.

P0375

The role of Galectin 3 in Con A induced liver injury

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Purpose/Objective: Concanavalin A (Con A) induced liver injury is an established murine model of T cell mediated hepatitis. We used this model to study the role of Galectin 3 (Gal-3) in the induction of inflammatory pathology and hepatocellular damage.

Materials and methods: We tested susceptibility to Con A-induced hepatitis in galectin-3-deficient Gal-3($^{-/-}$) mice and analyzed the effects of pretreatment with a selective inhibitor of Gal-3 (TD139) in wild-type (WT) C57BL/6 mice, as evaluated by a liver enzyme test, quantitative histology, mononuclear cell (MNC) infiltration, cytokine production, intracellular staining of immune cells, and percentage of apoptotic MNCs in the liver.

Results: Gal-3($^{-/-}$) mice were less sensitive to Con A-induced hepatitis and had a significantly lower number of activated lymphoid and dendritic cells (DCs) in the liver. The level of tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and interleukin (IL)-17 and -4 in the sera and the number of TNFα-, IFNγ-, and IL-17- and -4-producing cluster of differentiation (CD)4($^{+}$) cells as well as IL-12-producing CD11c($^{+}$) DCs were lower, whereas the number of IL-10-producing CD4($^{+}$) T cells and F4/80($^{+}$) macrophages were significantly higher in livers of Gal-3($^{-/-}$) mice. Significantly higher percentages of late apoptotic Annexin V($^{+}$) propidium-iodide($^{+}$) liver-infiltrating MNCs and splenocytes were observed in Gal-3($^{-/-}$) mice, compared to WT mice. Pretreatment of WT C57BL/6 mice with TD139 led to the attenuation of liver injury and milder infiltration of IFNγ- and IL-17- and -4-producing CD4($^{+}$) T cells, as well as an increase in the total number of IL-10-producing CD4($^{+}$) T cells and F4/80($^{+}$) CD206($^{+}$) alternatively activated macrophages and prevented the apoptosis of liver-infiltrating MNCs.

Conclusions: Gal-3 plays an important proinflammatory role in Con A-induced hepatitis by promoting the activation of T lymphocytes and natural killer T cells, maturation of DCs, secretion of proinflammatory cytokines, down-regulation of M2 macrophage polarization, and apoptosis of MNCs in the liver.

Poster Session: The Immunobiology of Epithelial Cells

P0377

Balancing the activation of minor histocompatibility antigen specific T cells in transplantation

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Purpose/Objective: T cell-mediated immune responses against vascular endothelial cells (ECs) represent a major obstacle in solid organ transplantation. A number of T cell-intrinsic and -extrinsic regulatory pathways impinge on chronic graft rejection. Here, we compared the impact of T cell-intrinsic negative regulatory circuits with T cell-extrinsic, regulatory T cell (Treg)-mediated attenuation during *in vivo* EC-T cell interaction.

Materials and methods: We used Tie2 mice expressing the β-galactosidase (βgal) antigen as a traceable minor antigen (mHA) under the control of the EC-specific Tie2 promoter. To model the interaction of ECs with specific T cells, we adoptively transferred βgal-specific $CD8^+$ T cells (Bg1) or $CD4^+$ T cells (Bg2) into Tie2 mice. To assess the impact of potential negative regulators Bg1 or Bg2 cells lacking the co-inhibitory molecules PD-1 or BTLA were used. To address whether Treg contribute to the control of EC-specific T cell activity, we used Tie2 × DEREG mice which facilitate ablation of Treg cells. Transplant rejection was assessed in a heterotopic heart transplantation model where Tie2 hearts are transplanted into B6 recipients.

Results: Bg2 and Bg1 cells were efficiently tolerized when the mHA was expressed systemically on ECs. Furthermore the lack of co-inhibition mediated by PD-1, BTLA or Treg cells did not prevent deletion of Bg1 cells, but differentially modulated initial Bg2 cells activation. Heterotopically transplanted heart grafts expressing the mHA in ECs remained immunologically ignored following transfer of mHA-specific $CD8^+$ T cells. However, ablation of the co-inhibitory molecules BTLA or PD-1 resulted in significant $CD8^+$ T cell activation leading to transplant vasculopathy. In contrast, mHA specific $CD4^+$ T cells induced vessel pathology in the presence or absence of negative regulation.

Conclusions: These data demonstrate that both T cell-intrinsic and -extrinsic negative regulation depends on the context of antigen presentation.

P0378

Distinct lympho-epithelial interactions control the segregation of IL-7-expressing cortical and aire-expressing medullary thymic epithelial niches

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Purpose/Objective: Thymic epithelial cells (TECs) are chief instructors of T cell development. Functionally distinct cortical (cTECs) and medullary (mTECs) TECs derive from a common bipotent progenitor and the partitioning of these subsets relies on reciprocal signals provided by thymocytes. While the molecular cues that control mTEC maturation are partially elucidated, the underlying basis that regulates cTEC differentiation is largely unknown, due to the lack of detailed intermediate stages. Using IL-7 reporter mice, in which YFP expression identifies TECs that co-express high levels of *Il7* ($Il7^{hi}$TECs), we previously showed that $Il7^{hi}$TECs numbers gradually decline with age in a thymocyte-dependent manner. Conversely, $Il7^{hi}$TECs are sustained when T cell development is profoundly blocked in $Rag2^{-/-}Il2rg^{-/-}$ mice, suggesting that $Il7^{hi}$TECs represent a TEC precursor controlled by thymocyte-derived signals. Here, we study the lineage relationship between $Il7^{hi}$TECs and other well-characterized TEC subsets.

Materials and methods: Immunocompetent and $Rag2^{-/-}$ IL-7 reporter mice were used. Fetal thymic analysis, *in vivo* anti-CD3-treatment and *in vitro* thymic organotypic cultures were performed. TECs were analyzed by flow cytometry and immunofluorescence. Gene expression was assessed by qPCR on sorted TECs.

Results: $Il7^{hi}$TECs emerge early during thymic development, continually retain a $CD205^+$ $BP1^+$ cTEC phenotype, co-express *Dll4*, *Ccl25*, *Il7*, *Ctsl*, *Psmb11*, *Prss16* and segregate from *Aire*, *Ctss* and *Tnfrsf11a*-expressing $CD80^+$ $CD40^{hi}$ mTECs. RANK and LtβR-mediated signals promote the maturation of mTECs. We show that the decline in $Il7^{hi}$TECs induced by thymocyte-TEC interactions dissociates from the RANK- and LtβR-mediated maturation of mTECs, suggesting that $Il7^{hi}$TECs are not a direct progeny of mTECs. Studies of $Rag2^{-/-}$ mice, in which mTEC differentiation is defective, reveal that $Il7^{hi}$TECs persist throughout postnatal life, indicating that their homeostasis is regulated by signals provided by thymocytes beyond β-selection. In anti-CD3-treated $Rag2^{-/-}$ mice, the induction of DP thymocytes lacking TCR expression restores mTEC differentiation without significantly curtailing $Il7^{hi}$TECs.

Conclusions: Taken together, our results indicate that high IL-7 expression is a determinant of cTEC lineage and provide evidence that the homeostasis of the cortical epithelium is regulated by thymocyte-derived TCR-mediated interactions.

P0380

IL-24 expression during early wound healing phase is depended on an inflammatory environment

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Purpose/Objective: Cutaneous wound healing is characterized by a precisely regulated action of different growth factors and cytokines. Its disturbance can lead to an impaired healing response, which represents a great medical problem. We demonstrated previously, that the novel IL-10 cytokine family members IL-22, IL-20, and IL-24, play a major role in psoriasis and skin homeostasis.

Materials and methods: Wound healing was investigated with an *in vivo* model using wildtype mice. Wound biopsies were examined by means of immunohistochemistry and quantitative real-time RT-PCR.

Results: By using an *in vivo* mouse model we demonstrated, that IL-20 was constitutively expressed, whereas no IL-22 expression was observed. In contrast, IL-24 was massively upregulated upon wounding. Here, immunohistochemistry revealed keratinocytes and CD3-positive cells as the main source of IL-24 in the early wound healing phase. In keratinocytes the IL-24 expression level was elevated after 24h stimulation with IL-4, IL-1b, IL-6, TNF-alpha and TGF-beta. We next investigated the expression of these mediators in non injured mouse skin as well as wound biopsies at indicated time points upon

wounding. T-cell derived cytokines were hardly induced in early wound healing phase. TGF-beta was constitutively expressed throughout the healing response. IL-1b, IL-6 and TNF-alpha were highly upregulated during the inflammatory phase and showed a similar expression kinetic as IL-24, which emphasizes a potential role for IL-24 upregulation. Interestingly, among these cytokines, IL-24 along with IL-1b were the highest expressed mediators relative to unwounded skin during the inflammatory phase.
Conclusions: IL-24 may have an important yet to be defined role in wound healing.

P0381

Molecular and cellular network in chronic lung inflammation and the impact of alveolar type II epithelial cells

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Purpose/Objective: For the development of therapies for chronic lung disorders, in depth understanding of the inflammatory processes are required. Therefore, the molecular and cellular network involved in the induction and regulation were characterized, with the specific focus on alveolar type II epithelial cells (AECII).
Materials and methods: Therefore, a transgenic mouse model for severe lung inflammation, based on the recognition of alveolar self-antigen by $CD4^+$ T cells, which closely resembles the pattern in human patients, was utilized. Cellular subsets in bronchoalveolar lavage (BAL) were compared using flow cytometric analysis. To characterize the inflammation-dependent changes on the AECII level, highly pure, primary AECII were isolated by flow cytometric negative selection. Transcriptional profiling was performed by Real-time RT-PCR. Furthermore, AECII were cultured and chemokines secreted by AECII as well as in BALF were detected using Proteome Profiler™ Array. To analyze the attraction of cells, *in vitro* migration was evaluated by transwell system. *In vivo* migration of CFSE-labeled $CD4^+$ T cells was evaluated at different time points using FACS.
Results: Analysis revealed an accumulation of immune cells in the inflamed airways. Protein profiling, in particular cytokines and chemokines, in bronchoalveolar lavage fluid (BALF) and AECII conditioned medium (AECIICM) from healthy and diseased mice showed inflammatory alterations. Inflammatory AECII revealed upon others elevated expression of co-inhibitory and co-stimulatory ligands which are known to pair with the corresponding receptors on activated T cells and regulatory T cells (Tregs) which have been shown to accumulate in the inflamed lungs. *In vitro* migration experiments revealed that soluble mediators in BALF and AECIICM have a specific and in part differential impact on the chemoattraction of neutrophils, dendritic cells and lymphocytes. *In vivo* $CD4^+$ T cell migration experiments revealed a dramatic accumulation of Tregs in the inflamed lung of recipient mice five days but not 1 day after adoptive transfer suggesting Treg expansion rather than attraction.
Conclusions: These data suggest an active contribution of inflammatory AECII to modulate autoreactive T cell responses and Tregs function. And a better understanding of the complex network composed of immune cells and soluble mediators which might determine the course of lung inflammation.

P0383

Polymorphonuclear neutrophil-derived elastase causes a dyshesion of pancreatic tumour cells and induces an epithelial-to-mesenchymal transition.

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Purpose/Objective: Pancreatic ductal adenocarcinoma (PDAC) is frequently associated with a scattered, disseminated growth pattern, an epithelial-to-mesenchymal transition (EMT), and a localised inflammatory response, evident as infiltration of leukocytes, particularly of polymorphonuclear neutrophils. In this study we explored a possible connection between the growth pattern, the EMT and the inflammation.
Materials and methods: Biopsies of patients with PDAC ($n = 112$) were analysed with regard to the leukocyte infiltrate, the nuclear expression of §-catenin and of ZEB1, both established indicators of EMT, and of E-cadherin, an intercellular adhesion molecule. Moreover, cultivated pancreatic tumour cells were co-incubated with isolated PMN, and the transition of the epithelial cells to cells with mesenchymal characteristics was assessed by time-lapse video microscopy, and by determining the induction of genes and proteins pertinent to EMT.
Results: We found in biopsies with a strong inflammatory infiltrate a nuclear accumulation of §-catenin and of ZEB1 in the tumour cells and a reduced expression of E-cadherin, all indicating an EMT within the tumour. To follow up on the question of a connection between PMN and EMT, pancreatic tumour cells grown as monolayers were co-cultivated with PMN. Within hours, a rapid dyshesion of the tumour cells was seen, and a drastic shape change to elongated, spindle-shaped cells. Subsequent experiments showed that dyshesion was mediated by PMN-derived elastase, which degraded the intercellular adhesion protein E-cadherin. Concomitantly with loss of E-cadherin, an up-regulation of TWIST, a gene involved in EMT, was seen, as was the translocation into the nucleus of §-catenin and of ZEB1, followed by a down-modulation of cytokeratin.
Conclusions: In conclusion, PMN-derived elastase induces a dyshesion and a transition of the pancreatic tumour cells to mesenchymal cells (EMT) *in vitro*. Because in pancreatic cancer the disseminated growth pattern and the transition to a mesenchymal phenotype coincide with a PMN infiltrate, a contribution of the inflammatory response to the induction of EMT and hence to tumour progression is possible.

P0384

Respiratory epithelial cell adhesion to type I collagen downregulates MMP-1 expression in TB

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Purpose/Objective: In tuberculosis (TB), Matrix Metalloproteinases 1 and 9 (MMP-1/9) have crucial roles in extracellular matrix (ECM) breakdown which leads to tissue destruction and cavitation. Pulmonary ECM is primarily composed of type I collagen.

We hypothesised that ECM components and specifically type I collagen regulate gene expression and secretion of MMPs. We investigated the effect of type I-IV collagen, fibronectin and laminin on MMP-1/9 expression and secretion in Normal Human Bronchial Epithelial (NHBE) cells stimulated with conditioned medium from Mtb-infected monocytes (CoMtb). We also dissected adhesion-dependent effects on tissue inhibitors of MMPs (TIMPs).
Materials and methods: NHBE cells were seeded in tissue culture plates pre-coated with ECM components and stimulated for 24 h with

CoMtb or control culture medium. MMP-1, 2, 7 and 9 and TIMP-1/2 secretion was measured by ELISA and Luminex bead array. mRNA concentration was detected by real-time PCR.
Results: CoMtb-stimulation of NHBEs adherent to type I collagen caused downregulated MMP-1 and upregulated MMP-9 concentrations compared to control cells adherent to tissue culture plastic ($P < 0.01$). Collagen concentrations of 100 μg/ml decreased MMP-1 from 786 ± 48 pg/ml to 557 ± 44 pg/ml (31% reduction), while MMP-9 increased from from 12 939 ± 1560 pg/ml to 21 571 ± 1767 pg/ml (40% increase). At the transcriptional level, there was a 41% decrease in MMP-1 mRNA and a 34% increase in MMP-9 mRNA. MMP expression by unstimulated cells was not affected by the presence of collagen ($P > 0.05$). No differences were found in secretion of MMP-2 or 7. Type IV collagen, fibronectin and laminin did not affect MMP-1 secretion. There was no difference in TIMP-1/2 concentrations between cells adherent to type I collagen or tissue culture plastic ($P > 0.05$).
Conclusions: Adhesion to type I collagen downregulates MMP-1 and 9 gene expression and secretion from CoMtb stimulated NHBEs. In contrast, TIMP-1 and 2 were unaffected. Therefore, MMP activity depends on ECM-cell interactions which might be important in the regulation of the inflammatory tissue destruction characteristic of TB.

P0385
Respiratory epithelial cells mediate distinct inflammatory responses to different bacterial pathogens

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Purpose/Objective: Streptococcus pneumoniae (Pn), Haemophilus influenzae (Hi), and Moraxella catarrhalis (Mc) are potential respiratory pathogens, which generally colonize the nasopharynx of humans early in life. Carriage of these bacteria decreases with age, but the immunological mechanisms functioning against colonization are largely unknown. IL-17 expressing Th17 cells have been found to mediate mucosal immunity to many bacterial pathogens. In this study we aimed to characterize the cellular immune responses Pnc, Hi and Mc trigger in human cells.
Materials and methods: Nasopharyngeal epithelial cells (Detroit 562) were incubated with live bacteria for 1 h. The proportion of adherent bacteria was determined by plate counting. During the next 23 h the epithelial cells were cultured with antibiotics to inhibit bacterial multiplication. Blood peripheral mononuclear cells (PBMCs) collected from healthy adults were incubated with heat-killed bacteria for 96 h. The cytokines (mRNA) expressed by the epithelial and PBM cells were measured by RT-qPCR.
Results: Exposure to live bacteria induced a rapid proinflammatory response in the epithelial cells; expression of IL-1α, IL-1β, IL-6, IL-8, and TNF-α, was increased but no significant differences were seen between cells exposed to different bacteria after the first hour. However, after 24 h, epithelial cells exposed to Mc or Hi expressed increased levels of all these cytokines, whereas cells exposed to Pn did not differ significantly from unstimulated cells. The proportion of bacteria binding to the epithelial cells was highest for Mc and Hi. Stimulation of PBMCs with Pn, in contrast to Mc and Hi, induced a marked IL-17 biased immune response coupled with down-regulation of IL-10. Mc induced a stronger IFN-γ mediated, Th1-type, proinflammatory response in the PBMCs than Pnc or Hi. Bacterial exposure also induced expression of Foxp3, typically considered a marker for regulatory T cells.
Conclusions: The results indicate that Pnc, Hi and Mc induce distinctly different types of immune responses in respiratory epithelial cells and T cells, suggesting that the host immune mechanisms against these pathogens may differ. Airway epithelial cells have the capacity to modulate the activation of immune cells, which means that the innate responses bacteria induce in epithelial cells can affect the adaptive immune response.

P0386
Role of epithelial E-cadherin in gut immune homeostasis

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Purpose/Objective: The transmembrane molecule E-cadherin is an essential component of epithelial junctions. It has emerged that it also plays a role in the immune system, as lymphocytes can express E-cadherin-specific receptors. $CD4^+$ $Foxp3^+$ regulatory T cells (Treg) are enriched for the E-cadherin receptors CD103 and KLRG1, suggesting a link between $CD4^+$ Treg and E-cadherin recognition. As gut Treg are key to prevent intestinal inflammation, we decided to assess the role of intestinal epithelial E-cadherin on gut Treg homeostasis.
Materials and methods: To check the effects of lack of intestinal E-cadherin on the immune system we used genetically modified mice. Complete lack of E-cadherin is not compatible with embryonic development, and specific deletion of E-cadherin from the intestinal epithelium also leads to prenatal death. To circumvent this problem, we replaced one allele of E-cadherin by the closely related molecule N-cadherin. The other allele of E-cadherin was specifically deleted on intestinal epithelial cells using a Villin-Cre E-cadherin flox system, so that E-cadherin positive cells throughout the body express both E- and N-cadherin but intestinal epithelial cells only express N-cadherin (gut E-Cad KO mice). We have then analysed the intestinal and systemic immune compartment from these mice using flow cytometry, microscopy and quantitative PCR.
Results: Unlike E-cadherin-sufficient littermates, gut-E-Cad KO mice show flora-dependent intestinal inflammation. This was not due to the ectopic N-cadherin expression as N-cadherin transgenic mice harboring one wildtype copy of E-cadherin showed no phenotype. These mice also presented increased $CD4^+$ T cell numbers in the gut. Interestingly, the frequency of Treg among the intestinal $CD4^+$ T cell population was also increased, mostly due to the accumulation of $Foxp3^+$ cells expressing the cadherin receptor KLRG1. The frequency of Treg expressing the E-cadherin receptor CD103 did not change significantly. As a control, we did not find increased $KLRG1^+$ Treg in the gut from IL-10-deficient mice, which also have flora-dependent intestinal inflammation.
Conclusions: Mucosal lymphocytes are controlled by local factors, and E-cadherin expressed by gut epithelial cells could be involved in the control of the intestinal Treg pool.

P0388
Soluble factors from alveolar epithelial cells increase intracellular killing using nitric oxide independent pathways

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Purpose/Objective: We have shown that factors from alveolar epithelial cells (AECs) increase macrophage intracellular killing of Bacillus Calmette-Guérin (BCG) through a nitric oxide-independent mechanism. We aim to identify a potential mechanism by which this increased killing is mediated.
Materials and methods: Bone marrow-derived macrophages (BMM) and pulmonary macrophages (PuM) were isolated from C57BL/6 mice

and infected with BCG. After a 4 h infection, bacteria were removed and cells incubated further in media with or without IFN-γ (20 ng/ml) or supernatants from AEC (AEC_{sup}). After culturing, intracellular bacterial killing was assessed, RNA was harvested and mRNA quantified using qPCR. Cytokines were also measured with ELISA.
Results: Treating BMM with IFN-γ, increased intracellular killing compared with that of cells cultured in medium alone whereas in PuM IFN-γ was ineffective. In both cell-types, IFN-γ treatment increased transcription of iNOS, IP-10 and IL-12 and secretion of IL-12 and IL-6. Transcription of suppressor of cytokine signaling (SOCS)1 was higher in PuM than BMM. SOCS1 expression was important for mediating the ineffectiveness of IFN-γ in increasing intracellular killing in PuM, as PuM from SOCS1/IFN-$\gamma^{-/-}$ mice showed enhanced killing after IFN-γ treatment. On the other hand, AEC_{sup} treatment did not affect iNOS, IP-10 nor IL-12 expression but increased Arg1 transcription and IL-6 secretion.
Conclusions: BMM and PuM responded to IFN-γ indicating that both cell types possess receptors able to recognize and transmit signals delivered by IFN-γ. The only difference was that IFN-γ only increased intracellular killing of BCG in BMM but not in PuM. On the other hand, treating cells with AEC_{sup} increased intracellular killing of BCG in both types of macrophages. This increased killing was not associated with increases in the pro-inflammatory effectors IL-12 or iNOS, indicating that the mechanism by which intracellular killing is increased after treatment with AEC_{sup} is not through an M1 activation pathway.

P0389

Synoviocytes change phenotype and function after Treg-depletion in arthritic mice

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Purpose/Objective: Immunization with Glucose-6-phosphate isomerase (G6PI) induces arthritis in susceptible strains of mice. Depletion of regulatory T cells (Tregs) prior to immunization switches the usually acute, self-limiting course to a non-remitting, destructive arthritis. This provides a good possibility to study relevant molecular switches for the transition from acute, self-limiting to chronic, destructive arthritis within one mouse model.

To examine the role of fibroblast-like synoviocytes (FLS), which can support and modulate immune responses via the production of pro- and anti-inflammatory mediators, the expression of a panel of surface markers characteristic of FLS was determined. Next, the phenotype and function of FLS from mice with either acute, self-limiting or non-remitting, destructive arthritis was studied.
Materials and methods: FLS from DBA/1 mice that developed either the acute or the chronic form of arthritis have been isolated from the joints over a time course of 56 days.To investigate the phenotype of FLS flow cytometric methods as well as quantitative realtime-PCR and ELISA studies have been performed. For the functional clarification of those cells the matrix-associated transepithelial resistance invasion (MATRIN) assay and a cartilage attachment assay have been used.
Results: It was found that murine FLS stably express several surface markers over several passages *in vitro*. Furthermore, FLS from Treg-depleted mice produced significantly more cytokines [e.g. Interleukin 6 (IL-6)] upon stimulation with other cytokines, growth factors and TLR ligands. This increased susceptibility to cytokine-stimulation in chronic animals compared to acute ones is observable throughout the disease course (56 days). Additional functional differences include the collagen-destructive potential and the potential to attach and eventually invade wild type cartilage. Here, FLS from Treg-depleted chronic arthritic mice showed a higher invasive and destructive potential.
Conclusions: Our results are compatible with the hypothesis that synoviocytes from Treg-depleted, arthritic mice acquire an autonomously aggressive phenotype that contributes to the switch from acute to chronic arthritis.

P0390

The regulatory effect of lactobacilli on *Staphylococcus aureus* induced inflammatory response in intestinal epithelial cells and immune cells

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Purpose/Objective: Dysbiosis early in life has been associated with immune mediated disease, such as allergy and inflammatory bowel disease. Previously we have shown differences in the early gut microbiota between children who developed allergic disease at five years of age and children that remained non-allergic. The mechanisms how the microbiota interacts with and influences the immune maturation after birth remain unclear. Here we aimed to investigate how different species of bacteria influence the response of the intestinal epithelial cells (IEC) and the immune cells.
Materials and methods: We exposed intestinal epithelial cell (IEC) lines (HT29 and SW480) to culture supernatants from seven *Lactobacillus (L.)* strains and three *Staphylococcus (S.) aureus* strains. Further peripheral blood mononuclear cells (PBMC) from healthy donors were stimulated with bacteria conditioned IEC supernatants. The level of cytokines and chemokines in the IEC and PBMC supernatants were analyzed qualitatively and quantitatively using human proteomic array and ELISA.
Results: The IEC lines produced a very limited set of cytokines and chemokines following bacterial exposure. Only *S. aureus* 161.2 induced an inflammatory response by the IEC characterized by the production of CXCL-1 and CXCL-8/IL-8. In PBMC, most of the tested *Lactobacillus* and *Staphylococcus* strains were able to induce IL-6 production, but only *S. aureus* 161.2 induced IFN-γ and IL-17. The simultaneous presence of epithelial factors did not significantly alter the response. Notably, the *S. aureus* induced IFN-γ and IL-17 production by PBMC, but not CXCL-8/IL-8 production by IEC, was down regulated by the simultaneous presence of any of the different *Lactobacillus* strains.
Conclusions: *S. aureus* 161.2 induced a strong inflammatory response in both PBMC and IEC, but there was a limited influence of IEC secreted factors on the PBMC response. Interestingly, Lactobacilli attenuated the *S. aureus* induced inflammatory response by immune cells, but not that of IECs, indicating a specific regulatory role on immune cells, although the mechanisms need to be further investigated.

P0391

The role of ceramide kinase in regulating LPS and TNF-alfa induced inflammation in A 549 cells

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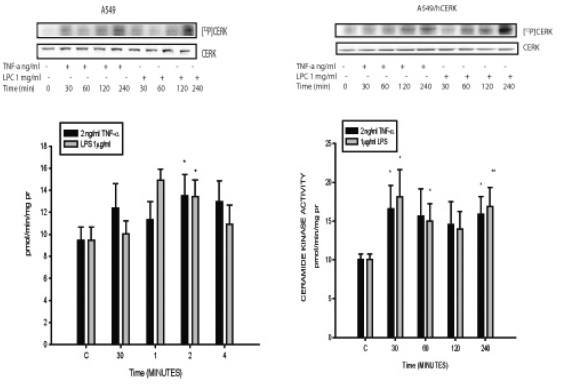
Purpose/Objective: Purpose: Activation of Toll-like receptors (TLRs) by specific pathogens leads to the production of proinflammatory mediators to initiate an inflammatory process. This is followed by a counter-regulatory, anti-inflammatory response to prevent excessive damage to the host. Tumor necrosis factor (TNF-α), one of the first cytokines to be released after activation of TLRs, is a key pro-inflammatory cytokine, and was reported that is down regulated by ceramide and its metabolite ceramide-1-phosphate (C1P)*(Rozenova K, et al., JBC, 2010; Lamour N, et al., JBC, 2011; Jozefowski et al, J Immun., 2010)*. Additionally, it has been reported that activation of TLR4 signaling by lipopolysaccharide (LPS) is dependent upon production of ceramide by acid sphingomyelinase (*Cuschieri et al., Surg Infect, 2007*). We reported recently ceramide-dependent PP2A regulation of TNF-α - induced IL-8 production in respiratory epithelial cells (Cornell T, et al., A. J. of Physiology, 2009). Objective: We are studying the regulation of inflammatory response through the action of phosphatases such as PP2A. Specifically, we hypothesized that in respiratory epithelial cells, LPS and TNF-α-induced inflammation is dependent upon activated ceramide kinase (CERK) and the resulting production of ceramide-1-phosphate (C1P).

Materials and methods: Methods: We used A549 cells stably transfected with hCERK. Additionally, cells were transfected either with siRNA duplex oligonucleotides targeting, CERK, PP2A or PP1 or with non-targeting siRNA duplex oligonucleotides (control) using Lipofectamine (Invitrogen).

Results: Results: Both LPS and TNF-α stimulation of A549 and A549/hCERK transfected cells increased CERK activity and also increased CERK phosphorylation. Mass spectrometry analysis revealed a significant increase of C1P. CERK activation impacted PP2A expression and activity, and led to changes in the regulation of Il-8 and TNF-α production. We also observed co-localization of CERK and PP2A by co-immunoprecipitation and by confocal microscopy using GFP-PP2A and RFP-CERK over-expression constructs.



The time-dependent formation of ceramide-1-phosphate (C1P) in activated A549 and A549/hCERK transfected cells. Cells were labeled with[^{32}P]H_3PO4 for 4 hours and then activated with 2nmol/ml TNF-α and 1mg/ml LPS. This increase CERK phosphorylation which correlated in parallel experiments with increases in intracellular C1P levels as measured by ^{32}P-labeling. Cells lysates were immunoprecipitaded with anti-hCERK antibody and examined by immunoblot using rabbit anti-phosphoserine antibody.

Conclusions: Conclusions: CERK appears to be a key enzyme involved in regulating inflammation by increasing C1P and influencing the activity of the regulatory phosphatase, PP2A. These studies were funded by AHA 0930039N.

Poster session: Ageing

P0395
Aged T cell subsets are characterized by a distinct microRNA signature

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Purpose/Objective: Decline of immunological responsiveness in elderly is at least in part attributed to changes in the composition of the T cell compartment. Recently, microRNAs (miRNA) have emerged as novel players in the regulation of T cell function. Little is known, however, on the expression of specific miRNAs and their role in altered T cell function with age. Aim of the study was to investigate the age associated changes in miRNA expression within defined T cell subsets in young and old healthy individuals.
Materials and methods: T cell subsets (naïve, memory, CD4 and CD8 cells) derived from young and elderly healthy subjects were sorted based on CD3, CD4 and CD45RO expression. RNA was isolated and miRNA expression patterns were determined for pooled T cell subsets ($n = 5$) using the agilent human miRNA microarray platform (V2) based on Sanger miRbase (release 10.1). Results were validated by qRT-PCR. A computational analysis was performed to identify miRNA putative targets and related molecular pathways.
Results: Hierarchical clustering showed differential expression of miRNAs mainly between naïve and memory subsets. Age related differential expression was observed predominantly within the naïve CD45RO$^-$ T-cell population. Seventeen miRNAs showed at least twofold up- or downregulation in aged naïve T cells. Analysis of individual samples revealed a statistically significant age related upregulation for miR-21, miR-223 and miR-451 by qRT-PCR. Computational analysis revealed putative miRNA targets associated with cell proliferation, apoptosis and insulin signaling pathways.
Conclusions: Age-related changes in miRNA expression are found predominantly within the CD45RO$^-$T cell compartment. It remains to be established if the differentially expressed miRNAs identified within the CD45RO$^-$ T cell subset in this study, converge with the accumulation of end-differentiated CD45RO$^-$ effector T cells re-expressing CD45RA.

P0396
Ageing alters the frequency and function of CD161$^+$ CD8+ T cells

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Purpose/Objective: Age-associated changes of immune function may affect recognition of pathogens, autoantigens and cancer cells. Ageing is associated with accumulation of T cells which express NK markers. Recently, circulating CD8+ T cells expressing the NK marker CD161 have been identified as potent producers of pro-inflammatory cytokines (IFN-gamma, IL-17) in young individuals. So far, little is known about the influence of ageing on CD161$^+$ CD8+ T cells. Therefore, we determined whether ageing alters the frequency and function of circulating CD161$^+$ CD8+ T cells.
Materials and methods: We assessed the relative and absolute number of CD161^{high} and CD161^{int} CD8+ T cells in peripheral blood from 62 healthy donors with increasing age (20−91 years). We analyzed intracellular expression of IFN-gamma and IL-17 by CD161^{high} and CD161^{int} CD8+ T cells from young (20−40 years) and old donors (>60 years) after stimulation with PMA/calcium ionophore + BFA. In the same donors, we also assessed intracellular expression of perforin and granzyme B in unstimulated CD161^{high} and CD161^{int} CD8+ T cells.
Results: In general, absolute numbers of CD8+ T cells decreased with age. Both the proportion and absolute number of circulating CD161^{high} CD8+ T cells decreased with age. Whereas the proportion of CD161^{int} cells increased with age, the absolute number of these cells remained stable. In contrast to CD161$^-$ CD8+ T cells, nearly all CD161^{high} and CD161^{int} cells produced IFN-gamma, regardless of age. CD161^{high} cells produced IL-17 and expressed the transcription factor RORgt. Interestingly, this potency to produce IL-17 was decreased in old donors. In general, CD161^{int} cells hardly produced IL-17. Both CD161^{high} and CD161^{int} cells expressed perforin. Although the majority of CD161^{int} cells produced granzyme B in young and old donors, granzyme B expressing CD161^{high} cells could be only detected in old donors.
Conclusions: We show that the frequency and function of CD161$^+$ CD8+ T cells is altered in non-pathological ageing. Firstly, CD161^{high}CD8+ T cells clearly decrease with age. Secondly, CD161^{high}CD8+ T cells produce less IL-17 in old individuals and become better equiped with cytotoxic effector molecules. How this affects the response to novel pathogens, autoantigens and cancer cells remains to be established.

P0397
Ageing shifts the Treg-Th17 balance

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Purpose/Objective: Ageing may affect various lymphocyte subsets, including regulatory T cells (Tregs) and T helper 17 (Th17) cells. Conflicting results on Treg numbers in young and old donors have been reported.Furthermore, whereas various studies in young donors have shown a remarkable plasticity between Tregs and Th17 cells, little is known about the Treg-Th17 balance in healthy elderly people. Therefore, the aim of our study was to determine whether ageing shifts the Treg-Th17 balance.
Materials and methods: We assessed the number of naïve (CD25^{int}CD45RA+) and memory (CD25^{high}CD45RA-) Tregs in donors with increasing age. Furthermore, we assessed the propensity for production of IL-17 by memory Tregs and the total CD4+ memory population in young and old donors.
Results: First, we found that ageing is associated with a decrease of circulating naïve Tregs. This loss of naïve Tregs in the elderly could be entirely attributed to the loss of CD31+ recent thymic emigrant Tregs. Unlike naïve Tregs, the number of circulating memory Tregs increased with age. Next, we found that a small fraction of memory Tregs was able to produce IL-17, but this was lower in the elderly compared to young donors. In fact, the total CD4+ memory population from older donors produced less IL-17 compared to young donors, and an increased memory Treg/Th17 ratio was observed in the elderly.
Conclusions: In conclusion, the number of circulating naïve Tregs gradually decreases with age, as less recent thymic emigrant Tregs are present in the elderly. In contrast, the combined observation of increased numbers of memory Tregs and decreased numbers of Th17 cells tilts the balance towards Tregs in healthy elderly people. This finding would be in line with previous studies indicating that anti-

inflammatory immune responses are essential to preserve health in old age by neutralizing the pro-inflammatory state that tends to develop later in life (inflamm-ageing).

P0399
Altered regulation of Lck in T cells with aging

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Purpose/Objective: Our aim was to study whther the tyrosine phosphatase SHP-1, a key regulator of T cell signal transduction machinery is, at least in part, responsible for the impaired T cell activation in aging.
Materials and methods: T cells separated from young and elderly subjects. T cell stimulated via TCR. Use of WB, confocal microscopy, FACScan, biochemical enzyme assays and thymidine incorporation assay.
Results: We showed that a dysregulation of the Csk/PAG loop in activated T cells from elderly individuals favored the inactive form of phosphorylated Lck (Tyr505). Dynamic movements of these regulatory proteins in lipid raft microdomains was also altered in T cells of aged individuals. We showed that SHP-1 activity was upregulated in T cells of aged donors compared to young subjects. Pharmacological inhibition of SHP-1 resulted in recovery of TCR/CD28-dependent lymphocyte proliferation and IL-2 production of aged individuals to levels not significantly different than those of young donors. Furthermore, we report differences in the active (Y394) and inactive (Y505) phosphorylated forms of Lck in response to T cell activation in elderly donors.
Conclusions: Our data suggest that the regulatory role of SHP-1 in T cell activation extends to its involvement in Lck-dependent negative feedback in aging. Modulation of SHP-1 activity could restore altered T cell functions in aging, suggesting a powerful tool for improvement of immunosenescence.

P0400
CMV-specific T cells with highly effector capacity are associated with 2-years all-cause mortality in healthy elderly individuals

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Purpose/Objective: To analyze CMV-specific T cell response in healthy elderly and its relatioship with human mortality.
Materials and methods: Sixty-seven subjects of the CARRERITAS cohort were selected according with the following inclusion criteria: (1) older than 50 years old, (2) without hospitalization during the last years, (3) without active infections, (4) without comorbidities or pharmacological treatment affecting the immune sistem and (5) positive IgG serology for cytomegalovirus (CMV) infection. T cell CMV-specific response was analyzed by multiparametric flow cytometry (IFNg, TNFa, IL2, MIP1a, CD107a and PRF1 production in response to the pp65 peptide pool).
Results: CMV-specific T cell response polyfunctionality was decreased in non-survivors. In addition, this subjects showed increased percentages of highly effector T cells expressing CD107a and perforine (CD107a+PRF1+) without IFNg coexpression. The CD107a+PRF1a+ subset was increased in both, CD4 and CD8 T cells and was correlated with higher percentages of CD57-expressing T cells. A multivariate analysis showed that percentages of CD8 T cells expressing CD107a+PRF1+ (but not CD4 T cells nor the age) were independently associated with time to death.
Conclusions: Aberrant accumulation of the CMV-specific CD8+PRF1a$^+$ CD107a+ T cell response is associated with lower human survival. This population could be a a predictive marker of a immune collapse.

P0402
Cytokine production by monocytes from elderly patients with Candida-associated denture stomatitis

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Purpose/Objective: This study aimed to evaluate the cytokine production by monocytes, challenged *in vitro* with *C. albicans*, obtained from peripheral blood of elderly denture wearers with denture stomatitis (DS), compared with elderly denture wearers without DS and elderly and young non-denture wearers.
Materials and methods: The isolated monocytes were cultivated in 24-well flat-bottomed culture plates, in the absence or presence of lipopolysaccharide (LPS) or heat-killed *C. albicans* ATCC 90028. After 18 h, the supernatant was collected and submitted to the enzyme-linked immunosorbent assay (ELISA) for determination of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), CXCL8, IL-1β, monocyte chemotactic protein-1 (MCP-1) and anti-inflammatory cytokines IL-10 and transforming growth factor-β (TGF-β).
Results: The results demonstrated, in general, changes in monocytes from the elderly with DS, as compared to other groups: lower spontaneous production of CXCL8 and MCP-1; lower levels of TNF-α, IL-6, IL-1β, CXCL8, MCP-1 and IL-10 after stimulation with LPS; and reduced production of TNF-α and IL-6 after stimulation with *C. albicans*. Comparing young and old, regardless of the presence of DS, the results revealed changes in the monocytes of the elderly: a lower production of TGF-β, spontaneous and after stimulation with *C. albicans*.
Conclusions: In conclusion, the dysfunctional *in vitro* production of pro- and anti-inflammatory cytokines by monocytes from elderly patients with DS may represent an additional aspect associated with susceptibility to the development and to the persistence of DS. Still, the dysfunction in monocytes of elderly groups, regarding the *in vitro* production of TGF-β, may represent aspects related to immunosenescence.

P0403
Cytomegalovirus (CMV) dependent and independent changes in the ageing of the human immune system: a transcriptomic analysis

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Purpose/Objective: Ageing is associated with profound decline of the immune capacity, which is manifested as increased morbidity and mortality. It has been proposed that chronic antigen stress, mainly from persistent CMV infection, may have a causative role in immunosenescence. The persistent CMV infection has for example been associated with accumulation of exhausted, non-proliferative CD8+ cells that lack the expression of costimulatory receptors CD27 and

CD28, a hallmark of decreased immune competence. However, the number of individuals affected by CMV increases with increasing age, so that the great majority of the elderly are affected by CMV. Thus it can be difficult to establish which ageing-associated changes are due to increased CMV prevalence and which are only due to increased chronological age. We now wanted to identify genes and pathways that are affected with ageing independently of CMV infection.

Materials and methods: We performed a transcriptomic analysis of peripheral blood mononuclear cells (PBMCs) with Illumina Human HT12 v4 BeadChip in a cohort of CMV seropositive (CMV+, $n = 140$) and seronegative (CMV-, $n = 6$) nonagenarians using CMV seronegative young individuals ($n = 11$, 19–30 years of age) as controls. Data was analysed with Chipster (CSC) and the affected pathways were identified with IPA software (Ingenuity Systems).

Results: Our results show that the gene expression profiles of $CMV^-$and CMV+ nonagenarians are different from each other. When comparing to the CMV^-controls we found 667 and 559 genes to be differentially expressed in CMV^- and CMV+ nonagenarians, respectively; 333 of these were common to both groups. Similarly, there were differences in canonical pathways affected; 45 and 16 pathways were affected in CMV^- and CMV+ nonagenarians, respectively, and 10 of the pathways were common. Interestingly, NK-cell and TCR-signalling pathways were affected only in the CMV^- nonagenarians. The up regulation of pro-inflammatory genes and pathways was however common to both groups.

Conclusions: Our data indicate that the CMV dependent and independent changes in the ageing of the immune system are fundamentally different. The results imply that inflamm-aging is independent from CMV infection, but that the cell-mediated immune functions, mediated by T cells and NK cells, are different depending on the CMV serostatus.

P0404

Effects of prolonged intense exercise on the adaptive immune response in elderly and young athletes

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Purpose/Objective: Exercise induces a series of changes on the immune system depending on their intensity and duration. In fact, transient states of immunosuppression are induced after intense physical activity and yet beneficial exercise anti-inflammatory effects have been described over many diseases and longevity.

Materials and methods: To study the impact of intense exercise for long periods of life on adaptive immunity at different ages we compared phenotypical and functional features of T lymphocytes of young ($n = 27$) and elderly athletes ($n = 15$) with young ($n = 30$) and elderly non-athletes ($n = 30$). We characterized leukocyte and lymphocyte subpopulations by flow cytometry and measured the T cell proliferation and activation response (CD69) against anti-CD3. We also studied the percentage of recent thymic emigrants (RTEs) by quantification of TREC by real-time PCR. Specific antibody titers against CMV were determined by ELISA.

Results: Leucopenia was found in both groups of athletes, mainly explained by low levels of neutrophils and lymphocytes. Exercise induced higher frequencies of NK, B lymphocytes and CD8+ T cells, whereas CD4+ T lymphocytes showed significant lower levels in the elderly athletes. Moreover, young athletes showed significant differences in all parameters that define the immune risk profile (IRP), with characteristics of an aging immune system, but we did not find differences between elderly groups. Less differentiated subsets of T lymphocytes were more frequents in non-athletes, with the exception of CD8+ T lymphocytes in young individuals. The analysis of TREC content in the elderly groups showed no significant difference in either the CD4+ or CD8+ T lymphocytes. In the young non-athletes group we observe an increase in the content of TREC in CD8+ T cells, but not in CD4+ T cells. Moreover, functional response of CD4+ and CD8+ T lymphocytes was significantly impaired only in young but no in elderly athletes.

Conclusions: Intensive training for long periods throughout life induces important phenotypical and functional changes on the adaptive immune response. These changes are most striking in young individuals and they damped with physiologic immune aging.

P0405

Intensity of the humoral response to CMV throughout the life configure phenotypical and functional status of the immune system in elderly

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Purpose/Objective: CMV infection exerts an enormous impact on human immunity being associated with an immune impaired response and also with a variety of chronic diseases and the overall survival in elderly individuals. History of CMV infection, and not only infection *per se*, may be determining the changes induced on the immune response.

Materials and methods: To study the impact of titers of anti-CMV antibodies on immune system we compared phenotypical and functional features of T lymphocytes of 92 healthy elderly donors and 70 young healthy controls. We characterized lymphocyte subpopulations by flow cytometry and measured the T cell proliferation and activation response (CD69) against CMV. We also studied the percentage of recent thymic emigrants (RTEs) by quantification of TREC by real-time PCR. Specific antibody titers against CMV were determined by ELISA.

Results: Titers of anti-CMV antibodies was used as a measure of accumulated antibodies throughout the life, and in fact titers were significantly higher in elderly and correlated positively with the specific CD4+ T cells responses to CMV. In elderly, the antibody titers were associated with the differentiation degree and the TREC content in CD4+ T cells, were correlated to other parameters belonged to the IRP and emerged as a conditioning factor over the ability to respond to immunization *in vivo*. Lack of correlations in young subjects may be due to lower anti-CMV antibody titers that they show. However, at similar levels of antibodies differences in highly differentiated and naïve T cells between young and elders were accentuated as titers increase. The reduction in absolute counts of naïve CD4+ T cells, also observed in individuals with higher titers, may be acting as a strategy to compensate the expansion of differentiated cells and to avoid the increase of total T cells.

Conclusions: In summary, our data show that titers of anti-CMV antibodies and not only CMV-seropositivity influence the differentiated status and the immunocompetence in the elderly, emerging as an important target in the improvement of the immune system function in elderly.

P0406

Long term injected with D-galactose altered the immune responses in C57BL/6J mice

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Purpose/Objective: Age-related changes of the immune system contribute to the increased susceptibility of elderly persons to infectious diseases. Mice chronically injected with D-galactose (D-gal) have been used as an animal aging model. However, less attention has been paid to the changes of systemic immune responses on D-gal induced aging mice.
Materials and methods: To investigate the changes of cytokines on D-gal injected mice. The C57BL/6J mice of model control (MC), Gal-100, Gal-150 and Gal-500 groups were subcutaneous injected with 0, 100, 150 and 500 mg/kg/day for 8 weeks. The brain and supernatants form immune cell culture were collected for cytokines assay.
Results: Our results indicated that Aβ were significantly increased in Gal-500 group, opposite to the activity of the mice. Furthermore, 150 mg/kg/day D-gal injected mice had higher cytokines production, especially TNF-α level in brain homogenates, but not in Gal-500 group. Moreover, D-gal injection toward decreased the IL-2 secretion, and increased the IL-4 production by splenocytes.
Conclusions: This study demonstrated that mice injected with 150 mg/kg/day D-gal could induce inflammatory cytokines production in brain, but not affect by 500 mg/kg/day injection group. There need more studies to confirm the mechanism of immune responses in D-gal induced aging mice.

P0407

Long term injected with D-galactose altered the immune responses in C57BL6J mice

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Purpose/Objective: Age-related changes of the immune system contribute to the increased susceptibility of elderly persons to infectious diseases. Mice chronically injected with D-galactose (D-gal) have been used as an animal aging model. However, less attention has been paid to the changes of systemic immune responses on D-gal induced aging mice.
Materials and methods: To investigate the changes of cytokines on D-gal injected mice. The C57BL/6J mice of model control (MC), Gal-100, Gal-150 and Gal-500 groups were subcutaneous injected with 0, 100, 150 and 500 mg/kg/day for 8 weeks. The brain and supernatants form immune cell culture were collected for cytokines assay.
Results: Our results indicated that Aβ were significantly increased in Gal-500 group, opposite to the activity of the mice. Furthermore, 150 mg/kg/day D-gal injected mice had higher cytokines production, especially TNF-α level in brain homogenates, but not in Gal-500 group. Moreover, D-gal injection toward decreased the IL-2 secretion, and increased the IL-4 production by splenocytes.
Conclusions: This study demonstrated that mice injected with 150 mg/kg/day D-gal could induce inflammatory cytokines production in brain, but not affect by 500 mg/kg/day injection group. There need more studies to confirm the mechanism of immune responses in D-gal induced aging mice.

P0410

Renal replacement therapy enhances CD8 T cell differentiation in young end-stage renal disease patients

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Purpose/Objective: End-stage renal disease (ESRD) patients have a defective T cell mediated immune system, which is related to excessive premature immunological ageing. The aim of this study is to examine whether this premature ageing is changed by renal replacement therapy (RRT).
Materials and methods: For this purpose, we studied circulating T cells of healthy individuals ($n = 60$), ESRD patients without RRT ($n = 30$, eGFR<15 ml/min), and patients treated with hemodialysis ($n = 30$) or peritoneal dialysis ($n = 30$). Groups were matched for age, sex and CMV serostatus. Three different assays were employed which are indicative for the immunological age of the T cell system. First, the T cell receptor excision circle (TREC) content was measured, which indicates the output of naïve T cells from the thymus. Relative telomere length (RTL) was determined as a measure for proliferative history and immunophenotyping was used to establish the differentiation status of circulating T cells.
Results: ESRD patients were significantly more affected ($P < 0.05$) compared to HC with respect to the parameters determined, i.e. ESRD patients had significant less TRECs ($P < 0.001$), shorter telomeres within both $CD4^+$ as well as $CD8^+$ T cells and were more differentiated towards memory T cells lacking expression of co-stimulatory molecule CD28. The percentage of pro-inflammatory $CD8^+$ CD28null T cells was significantly higher ($P < 0.001$) compared to HC. The $CD8^+$ T cells of younger patients on RRT have a significant ($P < 0.05$) shorter telomeres, a higher population of terminally differentiated $CD8^+$ T cells ($P < 0.01$) and a higher population of memory $CD8^+$ T cells that do not express CD28 ($P < 0.05$) as compared to the age related ESRD patients who did not receive RRT.
Conclusions: Based on the analysis of ageing parameters we conclude that the immunological age of T cells from ESRD patients is increased by on average 20–30 years compared to HC. RRT mainly affects the differentiation and proliferation of the $CD8^+$ T cell compartment in younger ESRD patients.

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P0411

Responsiveness of CD28-negative T cells to alternative costimulatory signals

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Purpose/Objective: Repeated stimulation of immune cells or persistence of pathogens results in a state called immunosenescene. Loss of CD28, the most potent costimulatory receptor on human T cells, is generally regarded to be a main predictor of biological aging of the immune system. Since CD28 negative T cells cannot receive signals via this potent costimulatory pathway, insufficient activating signals might contribute to the senescence state of these subsets. There are a number of other alternative costimulatory pathways, which can efficiently stimulate human T cells. Therefore we addressed whether alternative

costimulatory signals are able to overcome this senescent phenotype and restore immune function of CD28-negative T cells.
Materials and methods: Using an experimental system termed T cell stimulator cells we activatedCD28-negative CD8 T cells from elderly individuals in the presence of the costimulatory ligands CD166, ICOSL, CD70, 4-1BBL, MIC-A and CD58. Proliferative response and cytotoxic capability was analyzed.
Results: We found CD58 to generate the most potent costimulatory signal in these T cell subset.
Conclusions: The CD2-CD58 axis has an important role for the activation of CD28 deficient T cells.

P0413

Safranal ameliorates antioxidant enzymes and suppress lipid peroxidation and nitric oxide formation in aged male rat liver

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Purpose/Objective: Free radical production and oxidative stress are known to increase in liver during aging, and may contribute to the oxidative damage. The objective of this study was to observe the changes in activities antioxidant enzymes (superoxide dismutase, glutathione-S-transferase, catalase), lipid peroxidation levels and serum nitric oxide occurring in livers of rats of 2, 10 and 20 months age groups, and to see whether these changes are restored to 2 months control levels rats after administration of safranal.
Materials and methods: The aged rats (10 and 20 months) were given interapertoneal injection of safranal (0.5 mg/kg/day) daily for 1 month. We measured the activities ofantioxidant enzymes (superoxide dismutase, glutathione-S-transferase, catalase), lipid peroxidation levels and serum nitric oxide occurring in livers of rats of 2, 10 and 20 months age groups.
Results: The results obtained in the present work revealed that normal aging was associated with significant decrease in the activities of antioxidant enzymes, and an increase in lipid peroxidation in livers and nitric oxide content in serum of aging rats.
Conclusions: The results of the present study demonstrate that safranal could be a candidate to suppress the development of age-induced damages by protecting against oxidative stress and increasing antioxidant defenses.
Keywords: Safranal; antioxidant; liver; aging; rat

P0414

The butanol fraction of OAH19B ameliorates in monosodium iodoacetate-induced osteoarthritis

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Purpose/Objective: The aim of the present study is to investigate the potential therapeutic action of the OAH19B, a new herbal agent from butanol fraction of a mixture of *Aralia cordata* Thunb and *Cimicifuga heracleifolia*, on pain and cartilage damage compared with those of celecoxib in a monosodium iodoacetate-induced OA model.
Materials and methods: Experimental osteoarthritis was induced by the intraarticular injection of monoiodoacetate (MIA) into the right knee joints of rats.
Results: Both the oral administration of OAH19B dose-dependently and significantly inhibited cartilage damage in the knee joint and decreased the weight distribution deficit associated with MIA injection. OAH19B also significantly inhibited the degradation of GAG, while celecoxib did not affect cartilage destruction in the cartilage explants culture. OAH19B significantly decreased the levels of IL-1b, MMP-3, iNOS and COX-2 mRNA expression and immunostaining in the knee joints of MIA-injected rats. However, celecoxib decreased only the expressions of IL-1b and COX-2 mRNA and protein in the MIA-induced OA model.
Conclusions: In conclusion, OAH19B has both antinociceptive and cartilage protective effect by inhibitingproinflammatory cytokines and MMP-3 in the MIA-induced OA model. OAH19B, which was also more effective than celecoxib at preventing cartilage destruction, may be useful for the treatment of OA.

P0415

The immune responses of D-galactose injected C57BL/6J mice

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Purpose/Objective: Age-related changes of the immune system contribute to the increased susceptibility of elderly persons to infectious diseases. Mice chronically injected with D-galactose (D-gal) have been used as an animal aging model. However, less attention has been paid to the changes of systemic immune responses on D-gal induced aging mice.
Materials and methods: To investigate the changes of cytokines on D-gal injected mice. The C57BL/6J mice of model control (MC), Gal-100, Gal-150 and Gal-500 groups were subcutaneous injected with 0, 100, 150 and 500 mg/kg/day for 8 weeks. The brain and supernatants form immune cell culture were collected for cytokines assay.
Results: Our results indicated that Aβ were significantly increased in Gal-500 group, opposite to the activity of the mice. Furthermore, 150 mg/kg/day D-gal injected mice had higher cytokines production, especially TNF-α level in brain homogenates, but not in Gal-500 group. Moreover, D-gal injection toward decreased the IL-2 secretion, and increased the IL-4 production by splenocytes.
Conclusions: This study demonstrated that mice injected with 150 mg/kg/day D-gal could induce inflammatory cytokines production in brain, but not affect by 500 mg/kg/day injection group. There need more studies to confirm the mechanism of immune responses in D-gal induced aging mice.

P0416

Thymectomy in early childhood: immunosenescence in later life?

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Purpose/Objective: The study was aimed to investigate whether thymectomy in early childhood due to cardiac surgery may lead to alterations of peripheral naïve T-cells as found in immunosenescent elderly people.

Materials and methods: Forty-three patients who had thymectomy >15 years ago, 19 healthy, age-matched controls (HC) and nine HC aged >60 years were included. Indicators of T-cell-immunosenescence were studied: proportions of peripheral $CD45RA^+$ $CD27^+$ CCR7+ naïve T-cells, the T-cell-receptor-excision-circles (TRECs) as markers for recent thymic emigrants (RTE, $CD45RA^+$ $CD127^+$ CD31+), Ki67-expression as a marker of peripheral replication, T-cell-receptor (TCR) diversity and IL-7 as a proliferative cytokine for T-cells.

Results: Naive T-cells of TP were significantly decreased compared to HC but still higher than HC > 60 years. In TP, total counts of RTE were almost equal to HC > 60 years. Proportions of intestinal derived $CD127^+$ CD103+ naïve CD8+ T-cells were significantly increased in TP compared to HC and HC > 60 years. TP demonstrated significantly lower TRECs in naïve T-cells which correlated with time post thymectomy and a higher percentage of Ki67-expresssing naïve T-cells. IL-7 levels of TP were similar to HC > 60 years. TP showed less polycloncal TCR distribution than HC.

Conclusions: The findings indicate that changes of the peripheral naïve T-cell subset in TP may resemble the findings of immunosenescent elderly persons after thymic involution. The peripheral naïve T-cell-homeostasis after thymectomy is maintained mainly by extrathymic expansion of pre-existing naïve T-cells to compensate the diminished thymic output. The pathophysiological role of these alterations, such as infectious complications or autoimmunity in later life, has to be determined in long-term follow-up.

Poster Session: Antigen Receptors: Specificities, Repertoires & Functions

P0417
Characterization of three new HLA-A alleles

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Purpose/Objective: During the routine in the HLA laboratory is common to find some patients or donors who don«t exactly match with the databases of HLA. The characterization of new alleles is a very necessary task in the improvement on haematopoietic transplant, solid organ transplantation. In this report we describe the identification of three new HLA-A alleles found in three individuals typed in our lab.
Materials and methods: The new alleles were detected during routine HLA typing by SBT, whose results showed a mismatched position with a known allele in each case. All three HLA-A alleles were cloned and nucleotide sequenced in isolation.
Results: The first new allele is equal to A*68:02:01:01 but with one nucleotide change at position 127 in exon 2 resulting in a coding change at codon 19 (GAG'AAG) with the replacement of a glutamic acid (E) by a lysine (K). In the protein structure this amino acid residue is in a beta sheet in the α1 domain. The nucleotide sequence is available at Genbank, accession number JQ815887, andthe*WHO Nomenclature Committee* assigned the name A*68:92.

The second allele is identical to A*29:02:01:01 except for one nucleotide change in the 188 position in exon 2 resulting in a coding change at codon 39 (GAĈ GGC) with the replacement of an aspartic acid (D) by a glycine (G). This position codifies a residue located in a loop between two beta sheets of the α1 domain which interacts with the peptide and with the TCR. The nucleotide sequence is available at Genbank, accession number JQ815888, and the *WHO Nomenclature Committee* assigned the name A*29:38.

The last one is equal to A*23:01:01:01 with one nucleotide change in position 615 resulting in a silent mutation at codon 181 (CGĈCGT) which codifies an arginine. In this case this allele was found in a LAL patient who was candidate for haematopoietic stem cell transplantation and who shares the mutation with her father. The nucleotide sequence is available at Genbank, accession number JQ815889, and the *WHO Nomenclature Committee* assigned the name A*23:01:09.
Conclusions: We have described three new HLA-A alleles, found in different individuals: two of them carry amino acidic changes in the α1 domain, A*68:92 and A*29:38, while the last one shows a silent change, A*23:01:09.

P0419
IFI16 is an early sensor of Listeria monocytogenes DNA in the cytosol stimulating IFN-beta expression

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Purpose/Objective: Infection with *Listeria monocytogenes* (*L. monocytogenes*) induces a potent innate immune response. Induction of the immune response is dependent on bacterial secretion of listeriolysin O (LLO) which mediates the escape of *L. monocytogenes* to the cytosol after entry by phagocytosis. Previous studies have shown *L. monocytogenes* DNA and cyclic dinucleotide monophosphates secreted by bacteria as potent inducers of IFN-b.

IFI16 is a predominantly nuclear protein which distributes to the cytosol upon viral infection and UV irradiation. Recent studies show that IFI16 directly interacts with cytosolic DNA to induce IFNb.
Materials and methods: THP1, a non-adherent monocyte-like cell line differentiated into macrophage-like cells was used for all studies. *L. monocytogenes* homogenates were prepared by sonication of bacterial overnight cultures and treated with RNase or DNase before transfection into cells. Short-hairpin-RNA was used for knock-down of the intracellular DNA sensor IFI16. Co-localisation between bacterial DNA and IFI16 was investigated by confocal microscopy.
Results: In THP-1 cells *L. monocytogenes*-induced a potent IFN-b expression. This induction was dependent on bacterial escape into the cytoplasm. Short-hairpin-RNA knock-down of the intracellular DNA sensor IFI16 strongly reduced *L. monocytogenes*-induced IFN-b expression indicating a role of IFI16 in the sensing of *L. monocytogenes*.

Transfection with *L. monocytogenes* homogenates induced IFN-b expression in differentiated THP1 cells, and this was sensitive to DNase treatment of the homogenates. Furthermore, we observed *L. monocytogenes* DNA present in the cytoplasm during infection and this co-localized with IFI16 and the adaptor molecule STING.
Conclusions: Based on these results we conclude that IFI16 is a sensor of *L. monocytogenes* DNA in macrophages stimulating IFN-b expression.

P0420
MHC Class I-restricted recognition of Immunodominant viral or tumoral epitopes by human CD4 T cells

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Purpose/Objective: It is generally considered that MHC class I-restricted antigens are recognized by $CD8^+$ T cells whereas MHC-class II-restricted antigens are recognized by CD4 T cells. Several examples of MHC class-I restricted $CD4^+$ T cells have been reported but the functional relevance of MHC I crossreactvity of the CD4 T cell compartment has not been assessed yet. This has been addressed through the systematic analysis of human HLA-A*0201-restricted CD4 T cells directed against various immunodominant viral or tumoral antigens.
Materials and methods: *ex vivo* frequencies of human $CD4^+$ T cells specific for several viral or tumoral A2/peptide complexes were assessed through a pMHC multimer-based enrichment approach in immune or non-immune donors. MHC class I-restricted CD4 T cell lines and clones directed against (liste des *pitopes ELA, PGT, pp65, HCV1 É) were generated and analyzed for specificity, functional avidity and ability to recognize various target cell lines *in vitro*.
Results: $CD4^+$ T cells directed against every pMHC class I complexes tested were detected in all donors. CD4 T cell lines and clones recognized target cell lines *in vitro* in a peptide MHC-class I dependent but CD4 coreceptor-independent manner. While these cells were seldom overrepresented in immune donors, they expressed TCR of very high affinity and showed a different cytokine production profile compared to their CD8 T cells counterparts.
Conclusions: This study shows that MHC I-crossreactive CD4 T cells, though present in all individuals, are scarce and not usually engaged in adaptive immune responses against the corresponding pMHC class I complex. Nevertheless, these cells represent a natural source of high affinity TCR that could be exploited for TCR gene transfer strategies.

P0421

Recruitment and phosphorylation of the subsynaptic pool of LAT at the immune synapse requires the SNARE protein VAMP7

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Purpose/Objective: The LAT adaptor plays a crucial role in T cell antigen receptor (TCR) induced signalling. Two pools of LAT are present in T cells, one at the plasma membrane and one in intracellular sub synaptic vesicles. Recent results have shown that TCR activation results in the recruitment and phosphorylation of LAT from these sub synaptic vesicles. This raises the question of the mechanisms involved in the recruitment and fusion of these intracellular LAT pools. The purpose of this study was to characterize the mechanisms involved in the docking of the intracellular pool of LAT. We herein investigated the role of the vesicular SNARE protein, VAMP7, in the docking of LAT containing sub synaptic vesicles at the plasma membrane and in TCR signalling.
Materials and methods: The role of VAMP7 in LAT recruitment at the immune synapse was investigated using high resolution imaging techniques, including total internal reflection fluorescence microscopy (TIRFM), and photo-activatable localization microscopy (PALM). This was performed in Jurkat T cells silenced for VAMP7 expression using a lentivirus encoding two different shRNAs specific for VAMP7 and a control shRNA. Signalling in T cells was analyzed by Western blot analysis as well as by purification of signalosomes induced by TCR activation. Some of the results were reproduced in primary $CD4^+$ T cells from VAMP7 KO mice.
Results: Our results show that VAMP7 decorated vesicles are recruited and fuse at the plasma membrane of T lymphocytes upon TCR triggering. Moreover, they show that VAMP7 co-localizes with the intracellular pool of LAT and is required for LAT recruitment and phosphorylation at the immune synapse. Finally, analysis of the TCR induced signalling in T cells invalidated for VAMP7 expression reveals that the LAT containing signalosome induced by TCR triggering, does not form correctly but that yet some signalling occurs, i.e. ZAP70 is phosphorylated as well as SLP76.
Conclusions: Our data show that upon TCR triggering the VAMP7 SNARE protein controls docking and fusion of pre-existing intracellular pools of LAT at the immune synapse and that these events are required to induce the formation of signalling complexes. One implication of our results is that control of recruitment and fusion of endocytic compartments plays a crucial role in TCR signalling. Another implication is that the TCR signalling cascade is not linear but rather involved recruitment at the right place of components of the signalling pathway. These results thus suggest that imbalances in signalling regulation might be due to impaired intracellular trafficking of signalling molecules.

P0422

TCR or BCR CDR3 spectratypes differ between blood and spleen and between old and young patients, and can be used to detect myelodysplastic syndrome

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Purpose/Objective: Complementarity determining region 3 (CDR3) is the most hyper-variable region in B cell receptor (BCR) and T cell receptor (TCR) genes, and the most critical structure in antigen recognition and thereby in determining the fates of developing and responding lymphocytes. There are several millions of different CDR3 sequences of the TCR Vβ chain in human blood and about the same number of B cell clones. CDR3 length distributions (also called spectratypes) show variations between individuals and over time. However, the complexity of CDR3 length distribution patterns and the large amount of information included in each sample (e.g. 32 length distributions of the TCRα chain, and 24 length distributions of TCRβ) calls for the use of machine learning tools for full exploration.
Materials and methods: We have used supervised machine learning methods to analyze CDR3 length distributions from a variety of sources.
Results: We have found that the splenic B cell CDR3 length distributions are characterized by low standard deviations and few local maxima, compared to peripheral blood distributions. In addition, we show that healthy elderly people's B cell CDR3 length distributions can be distinguished from those of the young. Finally, we show that a machine learning model based on TCR CDR3 distribution features, mainly on TCRVβ22, can detect the myelodysplastic syndrome (MDS) with approximately 93% accuracy.
Conclusions: In summary, supervised learning models, based on a selection of distribution based features, may facilitate the use of CDR3 spectratyping as a monitoring tool for the health of the immune system.

P0423

The influenza nucleoprotein-specific memory B-cell pool is composed of multiple clonotypes with little clonal hypermutation

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Purpose/Objective: Immunization against influenza virus induces a pauciclonal expansion of antigen-specific B-cells. The resulting clonal plasma cell pools contain a highly diverse spectrum of somatically mutated cells. To examine how the antigen-specific B-cell diversity is maintained, we examined the repertoire of influenza nucleoprotein (NP)-specific memory B-cells 2 weeks after vaccination.
Materials and methods: Influenza NP-specific memory B-cells were isolated in a multi-step process from peripheral blood using protein-coated magnetic beads. The purity of the NP-specific B-cells was determined by ELISpot. Immunoglobulin G variable regions were amplified from single B-cells by RT-PCR, sequenced, cloned and expressed as recombinant monoclonal antibodies. Antigen specificity was determined by ELISA.
Results: Influenza NP-specific B-cell preparations were highly pure. More than 60 different clonotypes belonging to IGHV subgroups 1, 3, 4, 5, and 7 were identified in three subjects. The clonotype repertoire varied significantly between the subjects. Somatically mutated variants were detected for a minority of the clonotypes. Several memory B-cells had low numbers of somatic mutations.
Conclusions: Large numbers of different clonotypes and few somatically mutated variants indicate that the memory B-cell repertoire is primarily composed of different clonotypes rather than somatically mutated variants. Additionally, despite probable multiple exposures to influenza virus the vaccination induced several new influenza nucleoprotein-specific memory B-cell clonotypes in all donors.

P0424

Titer and avidity of IgG antibodies against Toxoplasma gondii and conventional PCR in patients with ocular diseases

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Purpose/Objective: To evaluate titers and avidity of IgG antibodies against *Toxoplasma gondii* and the presence of genomic DNA of *T. gondii* in peripheral blood of patients with toxoplasmic retinochoroiditis versus other ocular diseases.

Materials and methods: The sera of ocular disease patients were analyzed by indirect immunofluorescence, enzyme immunoassay and avidity tests and genomic DNA was investigated by conventional PCR. Statistical analysis used Fisher's exact test and the unpaired t test.

Results: Of the total sample, 65.7% (163/248) of patients were positive by ELISA and 34.3% (85/248) were non-reactive for IgG antibodies against *Toxoplasma gondii*. According to indirect immunofluorescence, titers of anti-*T. gondii* antibodies higher than 4000 were more common in patients with toxoplasmic retinochoroiditis compared to patients with other ocular diseases (8.1% versus 1.0%). No patients had IgM antibodies. Avidity of more than 60 prevailed in both groups. PCR identified genomic DNA more commonly in patients with toxoplasmic retinochoroiditis (21/62 — 33.9%) than in those with other ocular diseases (1/101 — 0.9%).

Conclusions: Toxoplasmic retinochoroiditis is common in patients with ocular diseases. Most patients with toxoplasmic retinochoroiditis have lower titers of highly avid IgG antibodies against *T. gondii*. PCR results suggest that *T. gondii* is found circulating in blood regardless of the presence of ocular lesions related to toxoplasmosis.

Poster Session: B Cells & Plasma Cells

P0425
A crucial function of the ligand-binding domain of Siglec-G, an inhibitory receptor of B1 cells

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Purpose/Objective: Siglec-G is a transmembrane protein of the Siglec family, which inhibits BCR signalling specifically on B1 cells. With it first extracellular Ig-like domain Siglec-G binds a2,3- and a2,6-linked sialic acids. These ligand interactions can take place to glycoproteins either on the same cell surface (in cis) or to ligands on other cell surfaces (in trans). We wanted to analyse whether the ligand-binding domain is important for the biological function of Siglec-G.
Materials and methods: We generated a Siglec-G R120E knockin mouse with a mutation in the critical amino acid involved in ligand-binding. We also developed a monoclonal anti-Siglec-G antibody, which did not exist so far, to study the Siglec-G expression pattern.
Results: Siglec-G R120E mice showed a large increase of the B1 cell population in the peritoneal cavity and in the spleen. B1 cells of these mice showed a better survival *in vitro* and expressed a changed BCR repertoire. Furthermore, Siglec-G R120E mice showed increased levels of serum IgM and increased $Ca2^{+}$ responses of their B1 cells. These phenotypes strongly resemble the phenotypes of Siglec-G-deficient mice. With our new anti-Siglec-G antibody we detected a quite uniform expression pattern on B cell subsets, therefore the B1-cell specific functions of Siglec-G cannot be attributed to a higher expression pattern on the surface of B1 cells, when compared to conventional B2 cells.
Conclusions: These experiments demonstrate an essential role for the ligand-binding of Siglec-G. We presume that the binding to sialic acid containing cis-ligands on the B1-cell surface regulates its inhibitory signalling function.

P0426
A role for IgG1 in immunity to Salmonella infection

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Purpose/Objective: The immune response to *Salmonella* infection initially is characterized by Th1 type cellular immunity, while germinal center and high affinity antibody development is delayed. Long-term protection from infection is mediated by humoral immunity. In mice the dominant Salmonella-specific antibody isotype is IgG2a, whereas IgG1 is only a minor component of the primary response. IgG2a dominance suggests that this isotype is most adapted to control challenge with *Salmonella*. Here, we have assessed whether IgG1 plays a significant role in immunity to *Salmonella typhimurium* (Stm) challenge.
Materials and methods: IgG1-deficient or wild-type mice were vaccinated with *Salmonella* outer membrane proteins. Five weeks later, mice were infected with high dose of Salmonella (SL3261). Complement dependent bacterial killing assay and phagocytosis assay were used to further detect the role of IgG1 for Salmonella infection.
Results: While wild type mice are protected at this stage, IgG1-deficient mice had much higher bacterial burdens in livers and spleens. Protection from *Salmonella* infection inversely correlated with the concentration of *Salmonella*-specific IgG1. This was not due to deficiencies in high affinity antibody production: Total antigen-specific IgG levels were similar to wild type mice, due to compensatory production of IgG2a and IgG3. Experiments with haptenated protein show that absence of high-affinity IgG1 is compensated by maturation of high-affinity antibody of other IgG subclasses. T cell memory and polarization of T cells in the early secondary response is also not affected, nor is the capacity to promote complement-dependent lysis. *In vivo* and *in vitro* experiments show that protection by opsonisation of *Salmonella* with immune serum and antibody mediated*Salmonella* uptake by macrophages is severely reduced.
Conclusions: IgG1 leads to compensatory Ig class switching to other affinity matured IgG classes, but these do not efficiently protect from Salmonella reinfection. IgG1 is mediating efficient opsonization and macrophage uptake, while no effect is seen from IgG1 deficiency on complement dependent lysis or on T cell responses.

P0427
A spontaneous mutation with low B cell phenotype discovered during the analysis of the function of Themis2 in B cells

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Purpose/Objective: Thymocyte-expressed molecule involved in selection (Themis) is expressed in T lymphocytes and has recently been reported to have a role in thymocyte positive selection. Themis2, sharing similar domains and high sequence similarity with Themis, is expressed in B lymphocytes, macrophages and dendritic cells. We hypothesized that Themis2 might have an analogous role in B cell development or activation to Themis in T cells and therefore created a Themis2-deficient mouse strain. During the generation of this strain we serendipitously found a spontaneous mutation leading to the loss of most mature B cells.
Materials and methods: We derived mice from a Themis2-targeted KOMP-CSD embryonic stem cell clone. These mice have been bred to recombinase bearing deleter-strains to obtain Themis2-deficient animals, which were analysed for Themis2 expression as well as defects in B cell development or activation.
Results: We show that Themis2 is expressed in all populations of B cells analysed, with lowest levels in germinal centre B cells and recently activated B cells. Redundant expression of other Themis family members was not found in any B cell population, either in wildtype or Themis2-deficient mice.

Analysis of Themis2-deficient mice has confirmed complete ablation of Themis2. Preliminary results indicate no defects in B cell development or activation or after immunisation with T-dependent antigens.

During the analysis of these mice however, we observed a defect in early B cell development in the bone marrow, which did not correlate with the Themis2 genotype. Further breeding experiments indicate that this defect is segregating freely from Themis2 with an X-linked recessive, Mendelian inheritance pattern. Sanger-sequencing of cDNA from these mice of the coding sequence of Bruton's tyrosine kinase, the most likely candidate for the spontaneous mutation, did not reveal any mutations.
Conclusions: The ubiquitous expression of Themis2 in the B cell compartment and its transcriptional regulation after activation suggests a yet unknown role for Themis2 in B cell biology. Currently, we plan to further analyse these mice for defects in immune responses or B cell activation in response to various, other stimuli. We will use exome sequencing in order to pinpoint the spontaneous mutation, which we have termed *atropos*.

P0428

Accumulation of circulating autoreactive naïve B cells reveal defects of early B cell tolerance checkpoints in patients with Sjögren's syndrome

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Purpose/Objective: Sjögren's syndrome (SS) is an autoimmune disease characterized by high affinity circulating autoantibodies and characteristic B cell disturbances with a predominance of naïve and a reduction of memory B cells in the periphery. It is unknown at what stages of B cell differentiation tolerance checkpoints are defective in SS. Here we aimed to determine the frequency of self-reactive and polyreactive B cells in the circulating naïve compartment of SS patients.
Materials and methods: Single IgD^+CD27^- naïve B cells were FACS sorted from 4 SS patients and RNA used to amplify Ig VH and VL genes which were then cloned and expressed as recombinant monoclonal antibodies displaying an identical specificity of the original B cells. B cells from two healthy donors (HDs) were used as control. Recombinant antibodies were tested towards different autoantigens to determine the frequency of autoreactive and polyreactive clones.
Results: A total of 66 individual recombinant antibodies were generated from naïve B cells of SS patients. Analysis of the VH and VL gene usage showed no significant differences between SS and HD. Our data showed increased reactivity towards ANA (43% SS clones versus 25% HD clones) and ENA (19% SS clones) but not increased polyreactivity in peripheral naïve B cells from SS patients, demonstrating an accumulation of autoreactive naïve B cells in the periphery of SS.
Conclusions: Here using a novel strategy to express recombinant antibodies from single B cells we demonstrated an elevated frequency of autoreactive naïve B cells in the circulation of SS patients strongly suggesting the existence of early defects in B cell tolerance checkpoints in SS.

P0429

Alpha-1-antitrypsin promotes B regulatory lymphocytes

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Purpose/Objective: B lymphocytes appear to play a role in autoimmune diabetes and also during islet allograft rejection. B regulatory lymphocytes, on the other hand, protect non-obese diabetic (NOD) mice from diabetes in an IL-10-dependent mechanism and also promote T regulatory cells. Alpha-1-antitrypsin (hAAT), an acute phase anti-inflammatory protein, induces immune tolerance during islet allograft transplantation in a mechanism that involves T regulatory cells. Recent findings suggest that B cells are required for hAAT-mediated protection of islet allografts.
Aim: Examine whether hAAT increases the proportion of B regulatory cells in culture and in various animal models.
Materials and methods: Splenocytes from GFP-IL-10 transgenic mice were added stimulatory concentrations of CD40 ligand ($CD40_L$), LPS and B cell activating factor (BAFF), in the presence or absence of hAAT (0.5 mg/ml). Cell-specific IL-10 production was evaluated by Flow Cytometry. GFP-IL-10 transgenic mice were treated with hAAT (60 mg/kg) and then grafted with allogeneic skin. The proportion of B regulatory cell population out of B cells was evaluated in draining lymph nodes (DLN) 7 days later by Flow Cytometry. Peritoneal cavity cell populations were assessed in hAAT transgenic mice ($hAAT^{+/+}$) and wild-type mice during non-sterile inflammation, as provoked by cecal puncture. All surgical procedures were SHAM-controlled.
Results: In cultures stimulated with $CD40_L$, LPS and BAFF, hAAT caused IL-10-positive B regulatory cell population size to increase 1.57 ± 0.06-, 1.5 ± 0.03- and 1.34 ± 0.04-fold, respectively ($P < 0.001$). In skin-grafted hAAT-treated mice, IL-10-producing B regulatory cell population size increased 3.06 ± 0.12-fold compared to skin-grafted untreated mice. $hAAT^{+/+}$ mice responded to non-sterile inflammation with a 3.56 ± 0.65-fold increase in peritoneal IL-10-positive B regulatory cells compared to wild-type mice.
Conclusions: B regulatory cells may play a role in the immunoregulatory activity of hAAT. Further studies are required to elucidate the mechanism of B regulatory expansion by hAAT.

P0430

Analysis of CD22 knockin mice with mutated ligand-binding and signalling domains

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Purpose/Objective: CD22 is a transmembrane protein, which mediates the inhibiton of the calcium signal after BCR crosslinking. The extracellular domain of CD22 can bind to its ligands α2,6-linked sialic acids, while the cytoplasmic domain contains inhibitory motifs. The purpose of this study was to analyse the ligand-binding and signalling domain of CD22 independently and thereby determine their functional interplay.
Materials and methods: We generated CD22-R130E *knockin* mice, which have a mutated binding pocket in the extracellular domain of CD22. These molecules are no longer able to bind their ligand α2,6-linked sialic acid. We also generated CD22-Y5,6F and CD22-Y2,5,6F mice with mutated ITIM-domains, so that the inhibitory SHP-1 phosphatase is no longer able to bind the CD22 cytoplasmic tail.
Results: In the bone marrow (BM) we found a reduction of recirculating B cells in the CD22-Y5,6F and CD22-Y2,5,6F mice similar to the phenotype of CD22-deficient mice. In contrast CD22-R130E mice did not show this phenotype. In the spleen all mice showed a reduced marginal zone B cell population. Similar to CD22-deficient B cells, only CD22-Y2, 5, 6F B cells showed increased turnover, as determined by BrdU incorporation *in vivo*. Ca^{2+} measurements revealed a decreased flux after BCR stimulation in CD22-R130E B cells, whereas the CD22-Y5,6F and CD22-Y2,5,6F B cells showed a higher Ca^{2+} flux. The CD22-R130E mutation affected the CD22 association to the BCR.
Conclusions: These data indicate that the maintenance of B cells in the marginal zone is dependent on the ligand-binding of CD22, as well as on the CD22 mediated signalling. However the reduction of recirculating B cells in the BM seems to be caused by the mutations in the ITIM domains of CD22. We could find a increased turnover rate, despite lower B cell numbers in the bone marrow and therefore conclude that B cells with defect signalling domains have a higher apoptose rate. These cells undergo apoptosis before they recirculate into the BM. The calcium flux after BCR crosslinking seems to be mediated by both the ligand-binding domain as well as by the signalling tail of CD22. In summary we found an important role for both functional domains of CD22 considering B cell homing, apoptosis and signalling.

P0432

B cell response to pneumococcal RrgA and RrgB antigens and its relationship with carriage in children and adults

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Purpose/Objective: *Streptococcus pneumoniae* remains a leading cause of childhood morbidity and mortality around the globe, even after three decades of 23-valent polysaccharide (PPV23) and one decade of 7-valent conjugate vaccines (PCV7) have been available in the market. Pneumococcal pilus plays an important role in their adherence to host respiratory epithelium. Pilus proteins (RrgA and RrgB) were found to be associated with increased bacterial colonization and invasiveness and a heightened inflammatory response in the host. We are investigating the B cell response to pilus proteins (RrgA and RrgB), developed in consequence to natural colonisation of pneumococcus;whether this priming can sufficiently induce immunological memory to protect against pneumococcal carriage and the potential of these proteins as mucosal vaccines in children.
Materials and methods: Adenotonsillar tissues, peripheral blood and nasal swab samples were collected from patients undergoing adenotonsillectomy. Nasal swab was cultured for pneumococcal carriage. Adenotonsillar mononuclear cells (MNC) were cultured with or without concentrated pneumococcal culture supernatants (CCS), to assess memory B cell responses by ELISPOT and to detect antibody responses by ELISA. Serum and salivary antibodies to pneumococcal RrgA and RrgB pilus proteins were measured by ELISA and by Western Blot.
Results: Preliminary results suggest that, pneumococcal colonisation is common in young children, which tends to decline with advancing age. The natural immunity to RrgA and RrgB proteins mount with progression of age. Anti-RrgA response seemed to develop earlier than anti-RrgB.
Conclusions: Serum antibody titres were higher in culture-negative children than those who were culture-positive for pneumococcus, suggesting that these antibodies are protective against pneumococcal carriage.

P0433

B-cell exposure to self-antigen induces regulatory B cells as well as IL-6- and TNF-a-producing B cell phenotypes in healthy humans

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Purpose/Objective: The ability of human B cells to secrete IL-10 after stimulation with self-antigens and to regulate self-antigen-induced T-cell responses in humans is still unclear. The present study was undertaken in order to determine whether exposure of normal human B cells to human thyroglobulin, a self-antigen with relevance to autoimmune thyroid disease, induced a regulatory phenotype or a pro-inflammatory phenotype in subsets of B or $CD4^+$ T cells.
Materials and methods: Thyroglobulin-pulsed B cells were isolated from healthy donors and co-cultured with autologous T cells. The production of IL-10 and TGF-β, in addition to the pro-inflammatory cytokines, TNF-α and IL-6 were observed and recorded.
Results: Pulsing with foreign antigen, tetanus toxoid, induced a Th1-response with minimal IL-10. Pulsing of B cells with thyroglobulin stimulated 1.10 ± 0.50% of B cells and 1.00 ± 0.20% of $CD4^+$ T cells for IL-10 production, compared to 0.29 ± 0.19% of B cells ($P = 0.01$) and 0.13 ± 0.15% of $CD4^+$ T cells ($P = 0.006$) following TT-pulsing. Thyroglobulin-stimulated IL-10 secreting B cells were enriched within $CD5^+$ and $CD24^{high}$ cells. While thyroglobulin-pulsed B cells induced only modest proliferation of $CD4^+$ T cells whereas B cells pulsed with TT induced vigorous proliferation.
Conclusions: Thus, B cells mediate self-antigen specific IL-10, TNF-α and IL-6 production in co-cultures with T cells and contributes actively to secretion of these cytokines.

P0434

B1 cells but not B2 cells exert regulatory activities through cell-cell contact independent pathway

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Purpose/Objective: B1 cells which are distinguished from conventional B2 cells belong to a special peripheral B lymphocyte subset and are mainly found in peritoneal and pleural cavities. Since B1 cells constitutively produce natural IgM in the absence of exogenous antigenic stimulation, they are thought to be parts of innate immunity. The basal level of IL-10 by B1 cells increases in response to stimulation with lipopolysaccharides or CpG. On the other hand, B2 cells could not produce IL-10 actively until they interact with T cells, stimulate with Ig and CD40, or contact with Toll-like receptor ligands. Based on the different characters of these two B cell populations, we speculate that B1 cells might play a regulatory role to suppress proliferation of T cells. In this study, we like to investigate further on the regulatory activities exerted by both B1 and B2 cells.
Materials and methods: We used wild type BALB/c mice and C.129P2 (B6)-*Il10*^{tm1Cgn}/J mice, which are IL-10 knock-out mice, to test whether $CD90.2^-CD5^+$ B1 cells and $B220^+$ B2 cells could suppress proliferation of $CD4^+$ T cells which are activated by anti-CD3 and anti-CD28 antibodies. Moreover, we used transwell experiment to clarify whether the immunosuppressive function of B1 cells is dependent on cell-cell contact. Naïve T cells were labeled with CFSE. These T cells activated by anti-CD3 and anti-CD28 antibodies cultured with B1 cells or B2 cells with transwell study or not. After 3 days, T cells were separated from B cells and analyzed by FACScan as well as [^{3}H] thymidine incorporation. The cultured supernatants were collected to measure the cytokine profiles by ELISA.
Results: Allogeneic stimulated proliferation of T cells was significantly suppressed by wild type B1 cells cultured supernatant. Neither wild type B2 cells cultured supernatant nor B1 and B2 cells cultured supernatant from IL-10 knock-out mice could suppress T cells proliferation. In addition, B1 cells could significantly inhibit T cells proliferation even with transwell experiment. The cytokines productions of T-B2 co-culture were IL-10, IL-2 and IFN-γ while the cytokines productions of T-B1 co-culture were abundant IL-10 and some IFN-γ.
Conclusions: These data suggested that B1 cells might play a regulatory role to suppress T cells proliferation by secretion of IL-10 and it is cell-cell contact independent. In the future, B1 cells might be studied further for their application to modulate immunological diseases.

P0435

Bcl3 overexpression leads to loss of marginal zone B cells

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Purpose/Objective: B cell homeostasis is regulated by multiple signaling processes including BAFF- B cell receptor and NF-kB signaling. Bcl-3 is a nuclear member of the IκB family that was originally identified as an oncogene in a subset of patients with chronic lymphatic leukemia. After nuclear translocation, Bcl3 associates with the NF-κB subunits p50 and p52 thereby enhancing cell proliferation and carcinogenesis through activation of cyclin D1 expression. Thus, Bcl-3 acts mainly as an oncogenic coactivator of NF-κB, although it is also able to repress NF-κB target gene transcription. Bcl-3 is required for lymphoid organogenesis and germinal center responses since mice deficient for Bcl-3 are immunodeficient due to microarchitectural defects of the lymphoid organs.

Materials and methods: We used a mouse strain allowing for the conditional and tissue specific overexpression of Bcl-3 upon Cre-mediated recombination. The coding sequence of Bcl-3 was inserted into the ROSA26 locus under control of the CMV early enhancer/chicken β actin promoter. Expression of Bcl-3 is prevented by a loxP flanked transcriptional STOP cassette. The compound overexpression of Bcl-3 and eGFP is permitted only after Cre-mediated recombination of the transcriptional STOP cassette in cells and tissues in which Cre is expressed.We used FACS analysis, ELISA and different immunizations protocols to investigate the role of Bcl3 overexpressin in B cells.

Results: Mice overexpressing Bcl3 appeared normal with a slight increased spleen at the age of 8 weeks. This increasement becomes more pronounced with age. FACS analysis revealed an accumulation of follicular B cells while the marginal zone B cells are absent. Further, B cells isolated from $Bcl3^{BOE}$ mice show reduced proliferative capacity upon a-IgM and LPS stimulation. But these B cells keep the ability to prolong survival *in vitro*. Moreover, T dependent immunization in $Bcl3^{BOE}$ mice revealed increased numbers of germinal center B cells.

Conclusions: Our results show a crucial role of Bcl-3 in survival of follicular B cells and terminal B cell differentiation.

P0436

Conjugate vaccination generates isotype-switched memory B-cells which depend on bystander T-cell help for their activation

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Purpose/Objective: Polysaccharide (PS) conjugate vaccines generate PS-specific memory B-cells (B_{MEM}) through the recruitment of $CD4^+$ T-cells, upon which the induction of B_{MEM} depends, via a conjugated protein (PT). However, in isolation the B_{MEM} thus generated appear unable to sustain immune protection following the waning of the initial serum antibody response. We have sought to establish whether this fundamental limitation of the conjugate vaccines reflects the ineffective activation of the B_{MEM} population as a result of the lack of cognate T-cell help subsequently available to them.

Materials and methods: A group of healthy adults ($n = 20$) who had previously been vaccinated routinely with a Men C conjugate vaccine were boosted with a Men C * tetanus toxoid (TT) conjugate vaccine. Men C and TT specific B_{MEM} in the circulation were enumerated using a memory ELISpot assay. The role of bystander T-cells and other non-cognate stimuli in driving the differentiation of B_{MEM} into Pc was subsequently established using antigen stimulation experiments employing both immunomagnetic cell separation and transwell experiments.

Results: Conjugate vaccination generated IgG^+CD27^+ B_{MEM} but not IgM^+CD27^+ B_{MEM} specific for Men C. We demonstrate that both the Men C and TT-specific B_{MEM}, generated following the administration of the conjugate vaccine continue to require $CD4^+$ T-cells in order to differentiate effectively into plasma cells.Nonetheless, bystander T-cells are able to provide such signals to the PS-specific B_{MEM} with comparable effect to the cognate T-cell help available solely to the TT-specific B_{MEM} population. Heat-killed meningococci drive the differentiation of the PS-specific B_{MEM} through bystander T-cell activation which is further enhanced by non-cognate T-cell independent innate signals. The bystander effects of T-cells activated by the conjugated TT within the vaccine were also demonstrated on B_{MEM} *in vivo*.

Conclusions: These data support the hypothesis that the differentiation of PS-specific B_{MEM}, at the time of bacterial encounter, depends on the capacity of bacterial PT to effectively activate bystander T-cells. Thus, priming such responses through including bacterial PT within conjugate vaccine preparations should be further evaluated.

P0437

CpG-ODN induces PD-L1 expression on human B cells and CpG ODN -treated B cells decreased IL-5 and IL-13 production from antigen-stimulated human $CD4^+$ cells

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Purpose/Objective: Co-stimulatory molecules are important for regulating T cell activation and immune response. Programmed death ligand 1 (PD-L1) also known as CD274 or B7-H1 has emerged as an important immune modulator that can block T cell receptor signaling. We investigated the effect of CpG-ODN on the human B cells *in vitro*. The expression of co-stimulatory molecule of B cells and its function were analyzed.

Materials and methods: B cells and T cells were separated from human peripheral blood mononuclear cells with magnetic beads. We have investigated whether PD-L1, and other costimulatory ligands could be expressed in human B cells stimulated by CpG-DNA. We sought to determine the effect of CpG-DNA-treated B cells on T helper 2 (Th2) cytokine production in Cry j 1 (Japanese pollen antigen)-stimulated human CD4-positive cells from patients with seasonal allergic rhinitis caused by Japanese cedar pollen.

Results: CpG-DNA strongly induced the coinhibitory molecule ligand, PD-L1 of human B cells. Results show that nuclear factor-kappa B (NF-κB) signaling is involved directly in CpG-DNA- induced PD-L1 expression in human B cells. CpG-DNA-treated B cells reduced Cry j 1-induced IL-5 and IL-13 production in CD4-positive cells. When the binding of PD-1 to PD-L1 was inhibited by PD-1-Ig, this chimera-molecule reversed the previously described reductions in IL-5- and IL-13-production. In contrast, the CpG-B-treated B cells increased both IFN-γ and IL-12 production in the presence of Cry j 1-stimulated CD4-positive cells. CpG-DNA simultaneously reduced the expression of B7RP-1 (also known as inducible costimulator ligand (ICOSL), B7-H2) and the ligand of CD30 (CD30L).

Conclusions: CpG ODN increased PD-L1-expression. CpG ODN -treated B cells decreased IL-5 production from antigen-stimulated human $CD4^+$ cells. These results suggested that the treatment of CpG-DNA suppressed antigen-specific IL-5 production via PD-1- PD-L1 ligation. This study reinforces the idea of CpG-DNA being a potential therapeutic modality through B cells and its signaling pathway being a target for drug interventions against allergic diseases.

P0438

Dietary omega-3 fatty acids induce a stronger B cell response in mice with antigen-induced inflammation

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Purpose/Objective: Omega-3 fatty acids may, in addition to their anti-inflammatory effects, affect resolution of inflammation. They are substrates for specialized pro-resolving lipid mediators that block production of pro-inflammatory mediators, inhibit infiltration of polymorphic neutrophils and stimulate phagocytosis by macrophages. The effects of omega-3 fatty acids on the adaptive phase of the resolution of inflammation has not been much studied. The objective of this study was to determine the effects of omega-3 fatty acids on the adaptive immune response in antigen-induced peritonitis.

Materials and methods: Female C57BL/6 mice were fed diets without or with 2.8% fish oil for 4 weeks. Peritonitis was induced by vaccination with mBSA in Freund«s adjuvant followed by an i.p. injection of mBSA. The mice were euthanized before and 1, 2, 5 and 10 days after mBSA injection. mBSA-specific antibodies in serum were determined by ELISA, peritoneal B cells analyzed by flow cytometry and germinal center B cells and mBSA in spleen evaluated by staining cryosectioned spleen sections.

Results: Germinal centers increased in size and number following administration of mBSA. There were more germinal centers and the germinal centers were larger in spleen from mice fed the omega-3 fatty acid diet than in mice fed the control diet. Serum levels of mBSA specific IgG and IgM antibodies increased slightly following mBSA administration. Mice fed the omega-3 fatty acids had higher serum levels of mBSA specific IgM antibodies than mice fed the control diet. However, serum levels of IgG were similar in the two dietary groups. There were more peritoneal B cells in mice fed the omega-3 fatty acid diet than in mice fed the control diet.

The amount of mBSA present in the spleen increased in both groups, reaching maximum 2 days after administration of mBSA. Mice fed the omega-3 fatty acid diet had less mBSA in their spleen on day 2 than mice fed the control diet.

Conclusions: These data indicate that the adaptive B cell response is more intense, with increased number of B cells, increased levels of mBSA specific IgM antibodies and increased number and size of splenic germinal centers in mice fed the fish oil diet than those fed the control diet. Therefore, dietary fish oil may enhance B cell response in antigen-induced inflammation and provide better protection in future encounters with the antigen.

P0439

Differential regulation of IRF4 during extrafollicular B cell differentiation, and its role for Ig class switching

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Purpose/Objective: The transcription factor IRF4 is essential for immunoglobulin class switch recombination (CSR) and plasma cell differentiation. Whilst high levels of IRF4 induce terminal differentiation to plasma cells, low-level expression of IRF4 is associated with CSR. Our aim was to study the kinetics of IRF4 induction and dissect the role of high and low-level IRF4 expression in CSR.

Materials and methods: To study IRF4 induction, we immunised quasi monoclonal (QM) mice, which have a high frequency of B cells specific for the model antigen 4-hydroxy-3-nitrophenylacetyl (NP), with NP conjugated to Ficoll (NP-Ficoll). This induces B cell activation with CSR, followed by parallel differentiation of both plasma cells and T-independent germinal centre B cells.

To test the role of IRF4 for Ig class switching in different phases of the antibody response, mice with NP-specific B cell receptors deficient in NFκB1 or overexpressing mir125 were produced.

Results: We show that low-level expression of IRF4 is induced within minutes of B cell activation. These become B blasts that undergo CSR. Three days after immunisation, B cells differentiate into germinal centre cells that lose IRF4 expression or extrafollicular plasmablasts. Plasmablasts express high levels of IRF4 and Blimp1, and do not undergo further CSR.

We show that whilst both NFκB1 and mir125b regulate IRF4 and plasma cell differentiation, they have different effects on the early phase IRF4 induction and Ig class switching in B blasts.

Conclusions: The results are compatible with a role for low-level expression of IRF4 for CSR in early B blasts.

P0440

Expression and functionality of proteinase activated receptor-2 (PAR-2) in murine B cells

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Purpose/Objective: PAR-2 is a seven transmembrane G protein coupled receptor (GPCR) that when activated by proteolytic cleavage results in the production of cytokines and chemokines(1). PAR-2 is expressed on numerous immune and non immune cells(2), however, it has previously been reported to be absent on B cells(3), although this remains controversial. The current study aims to address this controversy by demonstrating both PAR-2 expression and a functional role for PAR-2 in murine B cells.

Materials and methods: PAR-2 expression on murine B cells was assessed by flow cytometry. B cells were negatively enriched from C57BL/6 splenocytes using magnetic separation. Purified B cells were activated *in vitro* with the specific PAR-2 activating peptide SLIGRL-NH_2 (100 μM), supernatants collected 1, 4 and 24 h after stimulation, and analysed by ELISA for IL-6 and IL-10. Ca^{2+} signalling was measured by flow cytometry and pERK1/2 by western blotting. For sub-setting studies, spleens, bone marrow and peritoneal lavages were collected from female *F2rl1*$^{-/-}$ (PAR-2 deficient) and wild type C57BL/6 littermates, and cells stained with fluorescent antibodies for analysis by flow cytometry.

Results: Our data reveal that PAR-2 is expressed at low levels on splenic B cells, and this can be augmented by activation with SLIGRL-NH_2.The activation of B cells with SLIGRL-NH_2 also induced the increased secretion of IL-6 and IL-10. Furthermore, receptor activation induced Ca^{2+} mobilisation and the phosphorylation of ERK1/2 in B cells, indicative of intact PAR-2 signalling. Interestingly, the absence of PAR-2 in *F2rl1*$^{-/-}$ mice resulted in alterations in the composition of the B cell compartment, namely B1a and marginal zone B cells.

Conclusions: This study is the first to demonstrate the expression of functional PAR-2 on murine B cells, and implicates PAR-2 as a regulatory factor in the homeostasis of the innate-like B cell repertoire.

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P0441

Expression of Toll-like receptors 7 and 9 in B cell populations of patients with Sjögren's syndrome

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Purpose/Objective: Sjögren's syndrome (SS) is a rheumatic autoimmune disease, with focal lymphocyte infiltrations and inflammation in exocrine glands, resulting in destruction of glandular tissue. Primarily, salivary and lachrymal glands are affected, leading to reduced production of saliva and tears. A conse-quence of this is dry eyes and dry mouth (sicca syndrome). Other organs can also be involved in SS, and patients have, as many other rheumatic patients, major problems with fatigue. The cause of SS is so far not known, and there is no cure available. Only symptomatic treatment can be offered, and even if the inflammation is halted, it is not certain that the glands can be regenerated.

To generate new and more effective treatments for these patients, more information on the development and cause of this disease is needed. As SS is characterized by the production of autoantibodies, B cells have been recognized as important cells in the pathogenesis of SS and other rheumatic diseases. Toll-like receptors (TLR) are pattern recognition receptors that induce activation responses after ligand binding, and they are found on many immune cells. TLR7 and 9 are localized intracellulary in the endosomal compartment where they recognize nucleic acids. Both have been suggested to be involved in autoreactivity towards 'self'.

Materials and methods: In this study we utilized peripheral blood mononuclear cells to investigate the expression levels of TLR7 and TLR9 in various B cell subsets of SS patients and healthy controls using eight colour flow cytometry.

Results: Preliminary data suggests that memory B cells from SS patients have elevated expression levels of TLR 7 and 9.

Conclusions: Further analyses will be needed to evaluate possible functional differences of these cells.

P0442

FCGR3A-158V/F polymorphism may correlate with hypogammaglobulinaemia in patients after Rituximab treatment

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Purpose/Objective: Rituximab is a chimeric monoclonal antibody directed toward CD20, a B-cell surface marker that has been proven effective in depleting normal and malignant B cells *in vivo*. Rituximab is widely used in the treatment of B-cell malignancies and induces almost a complete depletion of normal B lymphocytes in peripheral blood for an average of various months. In some cases, Rituximab treatment causes prolonged hypogammaglobulinaemia. It has been reported that the presence of valine (V) at position 158 of FCGR3A (CD16) has a higher affinity to human IgG than the phenylalanine (F) allele. The hypogammaglobulinaemia after Rituximab treatment could be correlated with this polymorphism.

Materials and methods: This study initially included 16 patients who received treatment with Rituximab. The FCGR3A gene polymorphisms were determined by allele specific polymerase chain reaction (PCR). Genomic DNA was extracted from peripheral blood using a DNA isolation kit under the manufacturer's instructions.

Results: The patients tested for the FCGR3A-158V/F polymorphism were classified in low-affinity group (158 F/F) and high-affinity group (158 F/V and 158 V/V). Of the 16 patients initially tested for the polymorphism, 5 in Rituximab maintenance and 11 hypogammaglobulinemic subjects after Rituximab treatment, 3 patients had homozygous F/F, 11 had heterozygous V/F and 2 had homozygous V/V.

Conclusions: The aim of this study is to demonstrate the possible correlation between immunoglobulin levels after Rituximab treatment and the FCGR3A-158V/F polymorphism. In theory, patients classified in low-affinity group may have lower levels of immunoglobulins in comparison with high-affinity group. The confirmation of this result may imply the introduction of these studies as a diagnostic test and provide a more accurate Rituximab treatment to avoid secondary hypogammaglobulinaemia.

P0443

Functional elimination of double-stranded DNA-specific B Lymphocytes suppresses disease activity in SCID model of mouse lupus

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Purpose/Objective: Self-specific B cells play a main role in pathogenesis of Systemic lupus erythematosus (SLE). The elimination of B and T cells involved in the pathological immune response is a reasonable approach for effective therapy of SLE. In the present study we established an autoimmune model by transferring purified B and T cells from MRL/lpr mice to SCID mice and tested the effects of the chimeric molecule that selectively targeted pathological autoreactive B-lymphocytes.

Materials and methods: The protein chimeric molecule was constructed by coupling an DNA- mimotope peptide DWEYSVWLSN to a monoclonal anti-CD32 (FcγRIIb) antibody. This engineered molecule is able to cross-link cell surface immunoglobulin with the inhibitory FcγRIIb on DNA-specific B cells.

Female SCID mice were transferred with isolated $B^{+}T$ cells from MRL/lpr mice and the animals were treated with DNA- peptide chimera.

Cytokine assays, ELISpot, Signal transduction, Proliferation assay, ELISA and FACS analyses were also performed.

Results: The specific elimination of the DNA-specific B cells in MRL-transferred SCID mice restricts not only anti-dsDNA IgG production, but also autoreactive T cell activation and proliferation. In contrast, untreated MRL-transferred SCID mice experienced an increase of disease-associated antibody levels and developed glomerulonephritis similar to intact MRL/lpr mice.

Conclusions: In the present study we report a possible way to restrict the communication between autoimmune B and T cells, leading to suppression of lupus syndrome in MRL/lpr cell-transferred SCID mice. The functional elimination of autoantigen-specific B cells leaves autoreactive T cells alone without potency of prolonged pathogenetic effects.

In the present transferred SCID model of mouse lupus we have a possibility to study post-elimination disease processes and autoreactive T cell behavior after treatment with the protein-engineered antibody.

P0444

Gene profiling of human interleukin-10 producing regulatory B cells

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Purpose/Objective: B cells which possess regulatory function (Bregs) have been identified in mouse models of multiple sclerosis, rheumatoid arthritis and colitis. These Bregs are characterized by their ability to produce interleukin-10 (IL-10) which down-modulate inflammation. Similarly, we were able to detect IL-10 producing Bregs in human blood and spleen after *ex vivo* short time stimulation. Since IL-10 producing Bregs did not have a particular phenotype, a detailed profiling of this Breg subpopulation is needed in order to identify them more accurately based on unique cell surface markers and transcription factors.

Materials and methods: B cells were stimulated for 48 h with CpG and anti-Ig which are the most potent stimuli for inducing IL-10 secretion in B cells. Bregs were then isolated based on IL-10 secretion. Microarray analysis was performed in order to identify differentially expressed genes between IL-10 negative (IL-10-) and IL-10 positive ($IL\text{-}10^{+}$) B cell populations.

Results: Both naïve and memory B cells can secrete IL-10. Microarray analysis showed hierarchical clustering of B cells based on IL-10 expression. A total of 614 genes were found to be differentially expressed between the IL-10- and $IL\text{-}10^{+}$ B cell populations.

Conclusions: All subsets of B cells have the potential to produce IL-10 following CpG and anti-Ig stimulation. Our microarray findings may allow the identification of new subpopulations of human B cells with potential immunoregulatory function.

P0445

Generation of B-cell memory from the in-vitro-induced germinal center B cells

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Purpose/Objective: During T-cell dependent immune responses, antigen-bound B cells proliferate extensively to form germinal centers (GC) in the peripheral lymphoid organs and then differentiate into either long-lived plasma cells (LLPCs) or memory B (B_{mem}) cells, which constitute a B-cell part of the immunological memory. To understand the molecular mechanisms for the development of LLPCs and B_{mem} cells, we sought to construct an *in vitro* model system that recapitulates the GC reaction.

Materials and methods: Mouse naïve B cells were cultured on the feeder cells expressing CD40L and BAFF (named 40LB) with cytokines, IL-4 in the primary, and IL-21 in the following secondary culture. The B cells from these conditions were transferred into syngeneic mice, and after certain days, the mice were analyzed by flow cytometry, ELISA, and ELISPOT.

Results: In this culture system, the B cells extensively proliferated to generate germinal center-phenotype B (induced GC B: iGB) cells expressing switched or unswitched Ig classes. The iGB cells after primary culture with interleukin (IL)-4 develop into B_{mem} cells *in vivo* that elicit rapid immune responses in the presence of cognate helper T cells, whereas the secondary culture with IL-21 inhibits the B_{mem} development and allows their development into LLPCs that produce significant amount of IgG1 for more than one month in the bone marrow. The subsequent IL-21 withdrawal partially restores the B_{mem} development and abolishes the LLPC development. In addition, the transfer of the secondary iGB cells that are specific to a certain membrane antigen inhibited tumor formation of concomitantly transferred melanoma cells expressing the same antigen in the lungs of the recipient mice. Furthermore, we have established a system to selectively expand antigen specific iGB cells using the 40LB cells expressing a membrane-bound antigen and Fas-ligand.

Conclusions: This novel culture system has enabled *in vitro* differentiation from naïve B cells into B_{mem} or LLPC precursors, which can colonize and mature in their destined sites *in vivo*, and therefore will be useful to elucidate molecular mechanisms for the late B-cell development in GC. It also offers a model system to develop a B-cell-mediated anti-tumor therapy, in which patients will be transferred with autologous iGB cells selected for binding to a specific tumor antigen.

P0446

HA-specific memory B cell responses to pandemic 2009 H1N1 and seasonal influenza viruses in children and adults

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Purpose/Objective: Backgrounds: Influenza is a highly contagious and acute respiratory infection caused by influenza virus in the mucosa of respiratory tract. Nasal-associated lymphoid tissues (NALT) are mucosal immune organs in the nasopharynx, among which adenoids and tonsils are major components of human NALT.

Aims of study (i) to investigate whether prior exposure/infection of pandemic 2009 H1N1 virus (pH1N1) primes for immunological memory in human NALT and, (ii) To determine whether it cross reacts with seasonal H1N1 and H3N2 viruses.

Materials and methods: Adenotonsillar tissues were obtained from children and adults undergoing adenoidectomy and /or tonsillectomy due to adenoidal hypertrophy or tonsillitis. Adenotonsillar MNC were cultured in RPMI medium with or without the addition of influenza antigens. Peripheral blood samples were also taken for immunological analysis. HA-specific memory B cell responses to pandemic 2009 H1N1, seasonal H1N1 and H3N2 viruses were analysed by Elispot assay, following stimulation by surface antigens derived from respective influenza viruses. Serum anti-HA IgG antibody and haemagglutination inhibition titres were measured by ELISA and a standard HAI assay respectively.

Results: In individuals who had evidence of prior exposure to the 2009 H1N1 virus (who had anti-pH1N1 HAI titre $^{3}40$), there were significant numbers of pH1N1 HA-specific memory B cells which respond not only to the homologous pH1N1, but also cross-react to the sH1N1 viruses. However, there was no significant memory B cell response to the seasonal H3N2 HA after the pH1N1 antigen stimulation. Stimulation with the sH1N1 antigen induced only a moderate increase in the numbers of HA-specific ASC to the homologous sH1N1 and heterologous pH1N1, and no significant increase in numbers of specific ASC to sH3N2. In contrast, the pH1N1 antigen stimulation induced significantly higher HA-specific memory B cell responses, not only to the homologous pH1N1, but also to the heterologous sH1N1 than the sH1N1 antigen in patients with serum anti-pH1N1 HAI titre $^{3}40$. There was a significant correlation between numbers of HA-specific antibody secreting cells (ASC) to pH1N1 and that to sH1N1 after stimulation by the pH1N1 surface antigens.

Conclusions: Pandemic 2009 H1N1 influenza virus induces strong HA-specific memory B cell responses which cross react with the seasonal H1N1 but not seasonal H3N2 viruses.

P0447

Hypoactive Syk elicits B cell-mediated autoimmunity and a pre-diabetic state

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Purpose/Objective: The closely related non-receptor protein tyrosine kinases Syk and Zap-70 are of central importance in transducing signals from antigen receptors to downstream signaling mediators, a process ensuring proper selection, maturation and activation of lymphocytes. Partially overlapping protein expression patterns and reported functional redundancies at the signal transduction level still raise the question to which degree the kinases can replace each other in the immune system.
Materials and methods: To fully elucidate kinase interchangeability *in vivo*, we generated mice, which carry a Zap-70 cDNA 'knock-in' at the locus of Syk that is controlled by intrinsic Syk promoter elements and disrupts wildtype Syk expression.
Results: Kinase replacement strongly reduced Erk1/2-mediated survival and proper selection of developing B cells, demonstrating critical dependence on BCR signaling quality. The observed alteration in BCR signaling quality was accompanied by a preferential development and survival of marginal zone B cells, prominent knock-in serum autoreactivity, high levels of anti-insulin antibodies, proteinuria and an age-related glomerulonephritis. Development of concomitant fasting glucose intolerance in Zap-70 knock-in mice highlights aberrant B cell selection through hypoactive Syk as a potential novel risk factor for type 1 diabetes and suggests attenuated BCR signaling output as a mechanism to cause biased cellular and Ig repertoire selection.
Conclusions: Consequently, lowering Syk kinase activity in B cells through e.g. somatic mutation, alternative splicing / exon skipping or changes in promoter methylation might therefore contribute to autoimmune predisposition.

P0448

IL-2 induced MAPK-ERK pathway triggered human plasma cell differentiation through BACH2 Repression

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Purpose/Objective: Terminal differentiation of B lymphocytes into plasmocytes (PCs) involves a well-established transcription factor cascade. However, temporal dynamics of cell signaling pathways regulating transcription factor network during germinal center (GC) reaction remain poorly defined.
Materials and methods: To gain insight into the molecular processes and extrinsic factors required for B cell differentiation, we set up a controlled primary culture system combining BCR signal, Toll like receptor activation and T cell help in the form of CD40L and cytokines to differentiate human naïve B cells into PCs..
Results: We identify T-cell-produced IL-2 to be critically involved in ERK1/2 triggered PC differentiation. This early signaling event controlling specification to plasma cell fate operates independently of proliferation and survival functions. Compared to no cytokine activated B cells, IL-2-primed B cells display a gene expression profile of a more differentiated fate.Chemical inhibition of the MAPK-ERK pathway impairs IL-2 mediated PC differentiation and rescues the expression profile of several key genes, maintaining GC B cell fate. One of them encodes the transcription factor BACH2, which represses BLIMP1 the master regulator for PC differentiation, and whose expression is physiologically shutdown in centrocytes. Partial inhibition of BACH2 is sufficient to drive PC differentiation in absence of IL-2, and suppresses the effects of ERK1/2 inhibition on IL-2 induced PC differentiation as well. These results support the notion that the concentration of BACH2 fine-tunes the gene regulatory network involved in PC differentiation. The molecular mechanism of ERK-dependent BACH2 repression is under investigation.
Conclusions: Altogether we identify IL-2 as a novel early master regulator required to overcome the repressive forces that block PC differentiation through ERK activation and BACH2 inhibition, sustaining BLIMP1 expression.

P0449

IL-21 derived from human follicular helper T cells acts as a survival factor for secondary lymphoid organ, but not for bone marrow, plasma cells

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Purpose/Objective: OBJETIVE: IL-21 induces the differentiation of activated B lymphocytes into plasma cells (PC), but its direct effect on PC remains uncertain. This study analyzes the role of IL-21 on human *in vivo* -generated PC.
Materials and methods: MATERIAL AND METHODS: Expression of constitutive IL-21R on human PC from different organs was examined by flow cytometry. IL-21R mRNA from PC was quantified by RT-PCR. Tonsillar and bone marrow (BM) PC were purified by a combination of magnetic-selection and FCC-sorting. PC cultures without/with IL-21 or purified follicular helper T (Tfh) cells. Analysis of proliferating cells was performed by detecting BrdU incorporation. Apoptotic PC were determined by labeling active caspases with the fluorochrome-labeled inhibitor FAM-VAD-FMK. Measurement of Ig secreted to culture supernatants was performed by ELISA.
Results: RESULTS: IL-21R was clearly expressed on PC from human tonsil, lymph node and spleen (secondary lymphoid organs, SLO), but barely on terminally mature BM. PC. IL-21 enhanced Ig-secretion by isolated SLO PC, but not BM PC. Tonsillar Tfh lymphocytes are known to secrete IL-21. Purified Tfh-cells induced a marked increase of Ig-production by tonsillar PC, and this effect was impaired when endogenous IL-21-production was blocked. IL-21 provoked a rapid and transient phosphorylation of STAT3 in tonsillar PC. Tfh-cells or exogenous IL-21 reduced tonsillar PC apoptosis and increased PC recovery, but dit not modify their non-proliferating status.
Conclusions: CONCLUSIONS: These results suggest that IL-21 derived from Tfh-cells may act as a survival factor for SLO PC *in vivo*.

P0450

Immunoglobulin gene usage and specificity of plasmablasts generated during secondary dengue infections

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Purpose/Objective: Memory B cells generated after exposure to dengue viral (DENV) infection are a central component in shaping immune memory. Re-exposure to dengue virus of a different serotype is often associated with increased viral replication and disease pathogenesis. In repeated infection, the rapid activation of memory B cell leads to the generation of massive amounts of cross-reactive anti-

bodies. In the acute phase of a secondary infection, this B cell memory pool probably outcompetes the newly generated virus specific B cells.
Materials and methods: To characterize the specificity of plasmablasts and memory B cells generated during and after acute infection, we performed single cell RT-PCR for sequence analysis of Ig variable regions.
Results: Contrary to the polyclonal plasmablast repertoire we found that memory B cells isolated one month after disease were surprisingly oligoclonal and of different genetic composition than plasmablasts. Our current data revealed highly preferential usage of the VH1 gene in DENV binding plasmablasts. In contrast, skewed cell memory VH family repertoires (VH3) were noted in long lasting circulating cells isolated over a month after infection. Whether VH gene usages reflect a specific antigen/epitope selection is currently under investigation.
Conclusions: These data further suggest that plasmablasts represent a population of re-activated memory B cells with a potentially different origin than circulating steady state memory B cells.

P0451

Implication of proteins syntaxin-3 and syntaxin-4 in constitutive secretion of ig from human plasma cells

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Purpose/Objective: Plasma cells (PC) are B-lymphocytes terminally differentiated with the purpose of manufacturing and secreting immunoglobulins (Ig). This study explores the presence of several SNARE proteins in human PC and examines their functional roles on Ig secretion process.
Materials and methods: The U266 human myeloma cell line was used as model of Ig secreting cell line. Expression of constitutive SNARE proteins on human PC was examined by Western-blot and protein localization was analysed using immunofluorenscence confocal microscopy. Experiments of loss of function were performed by nucleofection of U266 with specifics iRNA. Overexpression assays were generated by transfection of constructs for native SNAREs and deleted protein lacking the transmembrane domain, both in fusion with Ruby as a reporter gen. Transfected cells, either iRNA o plasmid, were isolated by FACS-sorting and cultured for 24 h. IgE levels into the cultured supernatants were measured by ELISA and the corresponding cell pellets were used to analyse the inhibition or overexpression of each protein by Western-blot.
Results: Syntaxin-3 and Syntaxin-4 were detected in human PC and human cell line U266. The predominant location of Syntaxin-3 and Syntaxin-4 is on the cell surface but they could be also observed intracellularly. Although both Syntaxins shown to interact with SNAP-23, functional studies demonstrated a different role of these proteins in Ig-secretion. Interference of Syntaxin-4 expression, but not of Syntaxin-3, significantly inhibited Ig secretion in PC. Overexpression of wild-type Syntaxin-4 induced a marked decrease of Ig-secretion whereas overexpression of the mutant form of the protein did not modify significantly this secretion. Increase of both wild-type Syntaxin-3 and mutant form did not cause changes on Ig secretion levels.
Conclusions: The present study demonstrates that Syntaxin-4, but no Syntaxin-3 plays a critical role in the Ig-secretion and this data suggest that Syntaxin-4 along with SNAP-23 appears to be a component of the SNARE complex in human PC Ig-secretion.

P0452

Increase of IgA+, but not IgM+ memory B cells after vaccination with Pneumovax23

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Purpose/Objective: The mature B cell compartment is comprised of at least two B cell subpopulations, namely naïve follicular mature and marginal zone B cells. Whereas naïve follicular mature B cells are crucial during T cell dependent immune responses, marginal zone B cells are the main player during T cell independent immune responses. In humans, IgM+ memory B cells in the peripheral blood have been suggested to represent equivalents of splenic marginal zone B cells. However, this view remains very controversial.
Materials and methods: We therefore analyzed the changes in the composition of the B cell compartment in peripheral blood of healthy individuals following immunizations with Pneumovax23 inducing primarily a T cell independent immune response.
Results: Whereas we could not observe a significant change within the IgM+ memory B cell compartment we found a significant increase, both in relative and absolute numbers, in IgA+ memory B cells. Moreover, a 'non-responder' to vaccination, defined by no significant increase in Pneumovax-specific IgM or IgG, had very low, but still few, IgA+ memory B cells in peripheral blood.
Conclusions: Our data show, that Pneumovax23 primarily induces an IgA+ memory B cell reponse. Although it is well known that individuals with IgA deficiency show often normal responses to pneumococcal infection and vaccinations with Pneumovax23, these results suggest that IgA is primarily induced in response to these antigens and might serve as a predictor for the immune response.

P0453

Innate $CD19^+$ $CD45Rlo$ cell population exhibit high proliferation rates in homeostatic conditions and is able to respond after TLR stimulation

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Purpose/Objective: We described previously a novel splenic B cell population $CD19^+$ $CD45R^{lo}CD21^{lo}$ (19^+45R^{lo}) from embryonic origin, that preferentially home on perifollicular areas of unmanipulated follicles. These cells harbor a plasmablast phenotype (Blimp-1+Xbp-1+Pax-5+) and spontaneously release IgG1 and IgA. Here, we show that these cells are different from classical follicular, transitional, marginal zone B cells, B1 cells, the innate response activator B cells (IRA), and aged-associate B cells (ABCs).
Materials and methods: Multiparemetric flow cytometry analyses were performed on samples from different lymphoid organs. BrdU uptake and adoptive transfers of sorted $19^+45R^{lo}Ly5.1^+$ cells on $Rag2\gamma^{-/-}$ mice were performed. PCR array experiments from cell cycle genes were performed on sorted samples. IL6, IL10, IL12 and GM-CSF were quantified by RT-qPCR, magnetic bead arrays, and intracellular flow cytometer cytokine detection. Constitutive phosphorylation of p-ERK was also determined by flow cytometry studies. TLRs ligand stimulation, BAFF/IL4 and T-dependent stimuli were used for *in vitro* activation of 19^+45R^{lo} cells.
Results: 19^+45R^{lo} cells are present in Peyer's Patches, peripheral blood and spleen but not in lymph nodes or thymus. In the spleen they appear from 7 days post-natal age and are minimally present in CBA/CaHN (btk-). In homeostatic conditions, 50% of 19^+45R^{lo} cells are labeled after *in vivo* 17-day continuous administration of BrdU.

Purified 19^+45R^{lo}Ly5.1+ cells transferred on Rag2$\gamma^{-/-}$ mice survived up to 40 days. PCR array experiments performed on sorted 19^+45R^{lo} cells compared to 19^+45R^+ and to peritoneal B1 cells, confirmed an active cell cycling signature of 19^+45R^{lo} cells. *In vitro* stimulation of sorted 19^+45R^{lo} cells with LPS, CpG, and BAFF/IL4 induced cell proliferation and IgG1/IgA secretion. IL10-transcripts were found in resting 19^+45R^{lo} cells that also released IL10 after activation with LPS and BAFF/IL4. Finally, constitutive p-ERK1/2 was present in 19^+45R^{lo} cells, reflecting a pre-activation stage.

Conclusions: We conclude that 19^+45R^{lo} cells represent a novel component of the innate adaptive immune system with unique features, responsible of rapid IgG- and IgA-humoral responses. Additionally, we propose that 19^+45R^{lo} cells may play a potential immunoregulatory role in the initial phases of the encounters with common pathogens.

P0454

Interaction between B-1 cell and B16 melanoma cell: a mutual increase in cell survival

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Purpose/Objective: B-1 cells can be differentiated from B-2 cells because they are predominantly located in the peritoneal and pleural cavities and have distinct phenotypic patterns and activation properties. The role of both cell populations in cancer progression is still controversial. Previous work in our laboratory showed that physical interaction of B-1 cells with B16 cells *in vitro* leads to an increase in tumor cells metastatic potential. The aim of this study is to elucidate possible effects of this interaction on B-1 cells.

Materials and methods: Peritoneal B-1 cells were cultured alone or with B16 cells.B-1 cell survival and proliferation were evaluated by flow cytometry using propidium iodide and CFSE respectively. Considering that IL-10 is an autocrine growth factor that is used by B-1 cells to support their self renewal, we hypothesized that B-1/B16 cell interaction would increase the production of IL-10 by B-1 cells. The cytokine production was analysed by Th1/Th2/Th17 cytokines cytometric bead array (CBA) kit. Expression of STAT3, pSTAT3 and Bcl2 was assessed by Western Blot.

Results: B-1/B16 cells contact increased B-1 cells survival and proliferation *in vitro*. In order to clarify if contact between these two cell populations was necessary to increase B-1 cell survival; B-1 cells were cultivated on transwell which were placed on tumor cells or with B16-conditioned medium. In both conditions, it was observed an increment in B-1 cell survival. We also observed that after direct or indirect contact of B-1/B16 cells, B-1 cells became resistant to high dose of radiation. Analysis of cytokine secretion showed a strikingly increase in IL-10 production by B-1 cells after contact with B16 cells, associated with higher levels of STAT3 phosphorylation.

Conclusions: Our data clearly show that interaction of B-1 cells with melanoma cells, besides changing the tumoral lineage also affect B-1 cells, leading to both increased cell viability and rate of proliferation. Further, soluble factors that were secreted by B16 cells were sufficient to augment B-1 cell viability and IL-10 production. Impressively, after direct or indirect contact with the B16 cells, B-1 cells became resistant to radiation-induced cell death. Thus, future studies that assess the importance of concomitant immunity and other conventional therapies in cancer treatment are needed.

P0455

Interleukin-21-induced granzyme B-expressing B lymphocytes regulate T cells and infiltrate human solid tumors

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Purpose/Objective: B cells can exhibit potent regulatory functions. Conflicting data exist regarding the role of B cells in lymphocytic infiltrations of tumors. Here, we demonstrate, that granzyme B (GrB)-expressing B cells in adjacency to IL-21-providing T cells can be found in the tumor microenvironment of various solid tumors including breast, ovarian and cervical carcinomas. GrB-dependent regulation of T cell responses is known from both, regulatory T cells (T_{reg}) and plasmacytoid dendritic cells.

Materials and methods: Cell culture, FACS analysis, laser scanning confocal microscopy, Western blotting, histology.

Results: In the current study, we find that IL-21 induces human B cells to express high levels of GrB and to develop regulatory potential towards co-cultured T cells by GrB-dependent degradation of the T cell receptor z-chain. More detailed characterization of IL-21-induced GrB^+ B cells reveals a $CD19^+$ $CD38^+$ $CD1d^+$ $CD147^+$ phenotype and expression of further regulatory molecules including IL-10, IDO and CD25. Of note, $CD5^+$ B cells exhibit a significantly higher potential to express GrB than $CD5^-$ B cells, and GrB induction is accompanied by activation of both B cell receptor- and Toll-like receptor-associated signaling pathways.

Conclusions: In summary, we demonstrate for the first time, that IL-21 induces GrB-expressing regulatory B cells, which can be detected in the microenvironment of solid tumors and which may contribute to the modulation of cellular adaptive immune responses by T_{reg}-like mechanisms. Our findings may provide the basis for the development of novel diagnostic and cell therapeutic approaches to the management of malignant, autoimmune and graft-versus-host pathologies.

P0456

Interleukin-33 mediates activation and proliferation of effector and regulatory B cells

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Purpose/Objective: IL-33 is a novel IL-1 family cytokine with seemingly contrasting pro- and anti-inflammatory properties. Different types of immune cells express the IL-33 receptor ST2 and therefore can respond to IL-33 to initiate and regulate immune responses. Importantly, IL-33 has also been implicated in a wide range of inflammatory conditions such as allergic, autoimmune and cardiovascular diseases.

In the past, many IL-33 related studies have been performed using recombinant human IL-33 in murine systems. We have observed previously that human IL-33 induces CD25 expression and IL-10 production in murine B cells. During this work we aimed to further

characterize the potential pro- and anti-inflammatory properties of murine IL-33 on the activation and function of B cells.
Materials and methods: *In vivo* and *in vitro* B cell activation in response to IL-33 stimulation was studied by analyzing B cell functional markers by flow cytometry. IL-33 mediated B cell proliferation was investigated by CFSE dilution and thymidine uptake experiments.
Results: We report here that, although the murine cytokine is as expected significantly more potent, both human and mouse IL-33 induce B cell CD25 and IL-10 expression. The effect on B cell activation is largely independent of the genetic background of the mouse models tested. Interestingly however, mice of different backgrounds show significant differences in the IL-33 induced IL-10 production by B cells. We also found that IL-33 induces proliferation of splenic CD19+ B cells as well as upregulation of ST2 expression on their surface, suggesting that IL-33 may act on B cells directly. However, our findings from further *in vitro* experiments using isolated splenic B cells indicate that an indirect effect of IL-33 is also involved in driving the B cell responses. In addition, IL-33 induces upregulation of the low-affinity Fc receptor for IgE, CD23, as well as the costimulatory molecule CD86 on B cells.
Conclusions: Considering the diverse and crucial roles of B cells in both Th1 and Th2 immune responses, this work adds valuable information to our understanding of the immunological effects mediated by the novel cytokine IL-33.

P0457
Long-lasting and protective IgM antibody responses against Salmonella typhi

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Purpose/Objective: IgM antibodies can efficiently clear bacterial infections through the activation of the classical complement pathway. However very little is known about the mechanisms that govern the generation of long-lasting IgM. *S. typhi* porins are a candidate sub-unit vaccine that induces protective antibody responses. Here, we studied the mechanisms by which *S. typhi* porins induce a sustained IgM antibody response and the role of these antibodies in the protection against infection with *S. typhi*..
Materials and methods: Porins-specfic IgM antibodies were measured by ELISA and antibody-secreting cells were enumerated by ELISpot analysis. CD4 T cell responses were assessed following *in vivo* re-stimulation using intracellular cytokine staining. Porins-specific IgM hybridomas were generated and monoclonal antibodies used for *in vivo* protection assays.
Results: Boostered immunization with porins induced long-lasting, T helper cell-dependent IgM responses. Marginal zone B cell contributed to the first wave of IgM production after primary immunization, whereas a $CD4^+$ T cell-dependent germinal center reaction generated long-lasting memory B cells of the IgM type following booster immunization. Importantly, transfer of porins-specific IgM monoclonal antibodies protected mice from challenge with *S. typhi*.
Conclusions: In conclusion, long-lasting IgM memory B cell responses against porins represent an important layer of antibacterial protection against *S. typhi* infection.

P0459
Marginal zone B cells in autoimmune arthritis

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Purpose/Objective: Breakage of self-tolerance and induction of autoimmune arthritis is achieved in susceptible strains of mice by immunization with bovine (B) collagen type II (CII) in Freund's complete adjuvant (FCA). We have recently shown that naturally BCII-reactive marginal zone B cells (MZB) in the spleen expand rapidly following this immunization. Here we have investigated when and where true autoreactive B cells to murine (M) CII in relationship to BCII develop in collagen-induced arthritis (CIA), with emphasis on the MZB population.
Materials and methods: Cell suspensions were prepared from the spleen and peripheral lymph nodes from DBA/1 mice at different time points after BCII immunization. Mice immunized with ovalbumin (OVA) in FCA or FCA only served as controls. The B cell response was analyzed by flow cytometry and ELISpot. Serum was analyzed for antigen specific antibodies in ELISA.
Results: We show here that the initial autoimmune B cell response to MCII is induced and mediated by MZB in the spleen. The response is comparable to that towards BCII. One week later a B cell response to MCII appears in the lymph nodes. Notably, a small MZB-like population in the lymph nodes expand almost twofold after immunization with BCII but not with OVA or FCA. These cells are defined as $B220^{pos}CD23^{low}CD1d^{high}$ and are distinguishable from follicular B cells (FOB). The MZB-like cells are also $CD80^{high}Fc\gamma RIIb^{high}$ $MHCII^{low}$in contrast to FOB. IgM antibodies to MCII are found in serum a few days after BCII immunization with initially less titres than to BCII. In contrast, IgG antibodies to MCII are detectable at higher titres than to BCII about 2 weeks after immunization.
Conclusions: MZB trigger the autoimmune response in CIA. The autoreative B cell response in the lymph nodes develops later, involving FOB and the generation of IgG antibodies. The expansion of a MZB-like population in the lymph nodes is specific for the autoimmune reaction and may be of importance for the pathogenesis of CIA. However, the question remains whether these MZB-like cells have migrated from the spleen and whether they are CII-specific.

P0460
Oral immunization with T dependent antigen gives lifelong IgA immunity with long-lived plasma cells and memory B cells distinct the ones formed after systemic immunization

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Purpose/Objective: There is a discrepancy in the literature with regard to if mucosal immunization leads to long-term protection and development of memory. We reasoned that this could reflect differences between T dependent and independent responses and devised a system to address the response to a T dependent oral antigen.
Materials and methods: A strictly T cell dependent oral antigen was created by conjugating the hapten NP to the T dependent mucosal antigen cholera toxin to create NP-CT that was used for oral immunizations in mice.
Results: Repeated oral immunization resulted in strong antigen-specific IgA responses, with up to 15% of the gut plasma cells being NP-specific. Transfer of NP-specific GFP-labelled B cells prior to immunization demonstrated that the response was initiated in stomach-proximal Peyer's patches (PP) through invasion of pre-existing germinal centres (GC), then spread to more distal PP and finally to MLN and spleen. The presence of clonally related sequences

in different organs and between PP indicated that activated B cells could travel between germinal centres, a notion that was further strengthened by that transferred GC B cells could invade PP GC after iv transfer if antigen was present. Oral immunization with NP-CT resulted in secretion of specific IgA into the gut and IgA and IgG in serum for more than 2 years. A strong boost response 1 year after immunization demonstrated that memory B cells had formed. Transfer of GFP+ NP-specific B cells prior to immunization resulted in traceable long-lived class-switched memory B cells that expressed CD73, CD80 and CD273 and were situated in B cell follicles in PP, MLN and spleen. Oral priming followed by systemic challenge resulted in a strong response with rapid formation of plasma cells in the small intestinal mucosa, spleen and bone marrow, while systemic immunization followed by oral challenge did not generate any response. A difference in memory B cells formed after oral and systemic responses was further demonstrated in transfer experiment, where PP, and to lesser extent MLN, memory B cells preferentially produced IgA and spleen ones IgG in recipient mice.

Conclusions: We conclude that oral immunization with a T dependent antigen generates life-long immunity with long-lived plasma cells and memory B cells, and that there are distinctions between memory B cells formed after oral and systemic immunization.

P0462

Pro- and anti-inflammatory plasma cells can be distinguished by the expression level of sialyltransferase regulating IgG sialylation

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Purpose/Objective: The Fc glycosylation pattern determines the pro- or anti-inflammatory effector function of IgG antibodies, whereby sialylated IgGs are anti-inflammatory. However, how differentially glycosylated IgG antibodies develop is unknown. We sought to examine the Fc glycosylation of antigen-specific IgG molecules and plasma cells (PC) after induction of inflammation or tolerance.

Materials and methods: We administered chicken ovalbumin (OVA) under inflammatory or tolerance conditions to mice and analyzed OVA-reactive IgG Fc glycosylation and the expression level of the alpha 2, 6 sialyltransferase in OVA-reactive PCs.

Results: Stimulation with protein antigens under inflammatory conditions induces PCs expressing low levels of alpha 2,6-sialyltransferase and producing de-sialylated IgGs. In contrast, PCs induced upon tolerance failed to downregulate alpha 2,6-sialyltransferase expression and secreted immunosuppressive sialylated IgGs.

Conclusions: Our data show a novel antigen-specific immunoregulatory mechanism mediated by PCs expressing high levels of alpha 2,6 sialyltransferase and producing anti-inflammatory sialylated IgGs that are formed upon tolerance induction. Thus, pro- and anti-inflammatory PCs can be distinguished by the expression level of alpha 2,6 sialyltransferase.

P0463

Profiling B cell responses toward bacterial pathogens in human tonsils

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Purpose/Objective: The first contact between microorganisms and the human host takes place at the mucosal sites, in particular the upper respiratory tract and oro-pharyngeal mucosa. Data currently available on primary and recall B cell response in nasopharyngeal-associated mucosa are limited, due to the fact that more frequently the immune memory response to a pathogen or a vaccine is analyzed in PBMC isolated from peripheral blood. Therefore, the understanding of the mechanisms underlying the effector and memory response at the site of host-pathogen interaction is of great interest in the vaccine field.

The aim of the present study was to evaluate effector and memory B cells from palatine tonsils of patients that underwent surgery due to recurrent tonsillitis (RT) or obstructive sleep apnea syndrome (OSAS) by ELISpot assay.

Materials and methods: In order to avoid high background signal due to hyper-activated condition of B cells in tonsils, we have developed an *ad-hoc* protocol to enumerate simultaneously IgG and IgA antigen-specific B cells. The optimized protocol was applied to enumerate IgG+ and IgA+ plasmacells (PC) and memory B cells (MBC) specific for selected antigens of *S.aureus*, *S.pneumoniae* and Group A streptococcus.

Results: Frequencies of plasmacells and MBC were calculated as percentages of total IgG/IgA positive B cells in tonsils of 41 subjects aged from 4 to 42. Microbiologic culture data from tonsil swabs were also available for each analyzed sample.

Conclusions: We investigated if there were any differences in the antigen recognition profile between plasmacells and MBC of the same tonsil and tried to define a relationship between microbiological data and the pattern of the B cell response in tonsils.

P0464

Reassessment of immunoglobulin joining-chain expression during B cell development: an early marker of plasma cell differentiation in germinal centers

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Purpose/Objective: Immunoglobulin Joining (IgJ)-chain is a 15 kDa peptide expressed by antibody secreting cells and required for assembly of pentameric IgM and dimeric IgA. It is required for the transport of these polymeric immunoglobulins (pIg) to the mucosal surface. Despite this definite role, it still remains controversial to what extent plasma cells producing monomeric Ig isotypes also express this peptide. Likewise, the exact stage of IgJ-chain production during B cell differentiation into plasma cell is not clearly defined.

Materials and methods: To address this issue, we created a trangenic murine lineage that express the GFP under the control of the well-defined murine *IgJ* gene enhancer/promoter. This model allows us to follow IgJ-chain expression during *in vivo* and *in vitro* differentiation of B cells into plasma cells.

Results: We found that all plasma cells express a high level of GFP in every lymphoid organ observed including spleen and bone marrow. We confirmed that GFP positive cells also expressed IgJ protein. Interestingly, we detected a B cell subpopulation that express a lower level of IgJ-chain (GFP^{int} cells) in secondary lymphoid organs. Flow cytometry, immunohistochemistry and transcriptional analyses showed that these cells consisted in a large proportion of germinal center (GC) cells. However, Pax5 appeared to be slightly decreased in these GFP^{int} cells compared to resting B cells, while Prdm1 and Xbp1 expression are unchanged. *In vitro* stimulation of spleen B cells with LPS confirmed the expression of IgJ-chain in all $CD138^+$ plasmablasts but also revealed a population of $CD138^-/GFP^+$ activated B cells. ELISPOT experiments performed on these IgJ-chain expressing B-cells indicates these cells secreted larger amount of Ig than their IgJ-negative counterpart.

Conclusions: At first, this IgJ-GFP model is a reliable tool for PC tracking. Secondly, our results indicate that IgJ is already produced in

B-cells that seem to be pre-committed in the PC differentiation. Characterizing more precisely the features of this population would allow us to better understand early events of PC differentiation.

P0465

Regulatory B cells can modulate maturation and function of human dendritic cells and are partially defective in systemic lupus erythematosus

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Purpose/Objective: Mature dendritic cells (DCs) are stimulators of T-cell immune response, whereas immature DCs support T-cell tolerance. It has been demonstrated that murine B cells can hinder the inflammatory response by inhibition of IL-12 production by DCs. The current study was aimed at determining the regulatory capacity of human B cells on DC maturation and function.
Materials and methods: Peripheral blood monocytes from six healthy donors (HDs) and six systemic lupus erythematosus (SLE) patients were differentiated into immature DCs with GM-CSF and IL-4, and into or mature DCs with LPS and IFNγ. B lymphocytes were activated by CD40 and TLR9 and cultured with DCs. Expression of HLA-DR, CD80, CD86 and IL-12 by the DCs were evaluated by flow cytometry. The DC-dependent proliferation of CFSE-labelled T cells was also evaluated in the presence of B lymphocytes. Phenotypic analyses of B cells and inhibitory experiments were performed in co-cultures.
Results: Activated B lymphocytes restrained the development of monocytes into immature DCs and their differentiation into mature DCs. They decreased the density of HLA-DR from mature DCs, the expression of CD80, CD86, and also the production of IL-12 Furthermore, they inhibited the DC-induced T cell proliferation. These modulations were mediated by $CD19^{+}IgD^{low}CD38^{+}$ $CD24^{low}CD27^{-}$ B lymphocytes and needed CD62L for the control of CD80 and CD86, and a soluble factor for the control of IL-12. Mature SLE DCs were insensitive to the regulation of IL-12.
Conclusions: Human B cells can regulate DC maturation and function and consequently T cell-dependent inflammatory response. This regulation is partially deficient in SLE due to refratory DCs. This may influence inappropriate balance in SLE between effector inflammatory response and tolerance induction.

P0466

Regulatory role of B-1 cells in streptozotocin-induced diabetes in mice

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Purpose/Objective: Recently, an important role of B cells in promoting type 1 diabetes has been demonstrated. Concomitantly, it has also been shown that B-1a cells, a subtype of B lymphocytes, can be involved in various autoimmune diseases. Based on these data, this study aims to investigate the involvement of B-1 cells in the development of murine streptozotocin (STZ)-induced diabetes.
Materials and methods: BALB/c and BALB/*xid* (B-1 cell-deficient mice) male mice strains were treated intra-peritoneally with STZ (40 mg/kg) for 5 days. It is important to note that BALB/c mice do not develop diabetes when this dose of STZ was used. Blood glucose levels (BGL), cellular profile in the peritoneal cavity and cellular pancreatic islet infiltration were evaluated in experimental and control animals by flow cytometry. Further, pancreas were processed and analyzed histologically or immunohistochemically for insulin expression.
Results: Our findings showed that BALB/*xid* mice become diabetic after STZ treatment with BGL higher than BALB/c control mice ($P < 0.001$). Corroborating this data, the amount of insulin labeled beta cells of STZ-treated BALB/*xid* mice was always lower than control groups. Surprisingly, B-2 population increase in the peritoneal cavity of STZ-treated mice 10 days after treatment ($P < 0.001$) while $CD4^{+}$ T cells increase only in BALB/*xid* diabetic mice ($P < 0.01$). Histological analysis showed lower number of pancreatic islets in diabetic mice (BALB/*xid*) as compared with pancreas in BALB/c mice. Additionally, the cellular evaluation of pancreas showed infiltrating T cells in these mice. To evaluate the role of B-1 cells in diabetes induction, peritoneal B-1 cells obtained from BALB/c mice were purified based on expression of $CD19^{+}$ $CD23^{-}$ by FACSAriaII Cell Sorter and adoptively transferred intra-peritoneally to BALB/*xid* mice before or after the STZ treatment. BALB/*xid* mice adoptively transferred were not diabetic after STZ-treatment and B-1 cells were observed infiltrating pancreatic islets 2 days after STZ treatment.
Conclusions: Our data demonstrate that B-1 cell-deficient mice showed higher reactivity to STZ treatment with more severe symptoms, intensive pancreas damage, insulin deficiency and high BGL. In addition, B and T cells increase in peritoneal cavity of BALB/*xid* STZ-treated mice while B-1 cells migrate to pancreatic islets of non diabetic mice.

P0467

Revealing the role of Akt

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Purpose/Objective: The serine/threonine kinase Akt is expressed in three isoforms (Akt1, Akt2 and Akt3) which share a similar structure and analogue functions. Using distinct downstream pathways they regulate cellular metabolism, cell survival and proliferation, being important for peripheral B-cell maturation and early stages of T cell development. In B cells Akt is a critical check point in different phases of proliferation and differentiation. Especially the generation of marginal zone and B1 B cells depends on Akt1 and Akt2. In this study we investigated in more detail the role of Akt in B cell development and maturation.
Materials and methods: T. Wunderlich created a mouse allowing for the expression of an N-terminally myristoylated murine AKT carrying in addition a C-terminally attached Myc TAG (ROSA-AKT-C) in cell types that express the Cre recombinase (unpublished). The myristoylation signal recruits AKT-C to the plasma membrane where AKT becomes phospho-activated. This mouse was crossed to CD19 Cre mice leading to a B cell specific overexpression of Akt (AKT^{BOE}). For FACS analysis of $Akt^{BOE}$and CD19 Cre control mice we isolated cells from bone marrow, spleen, lymphnodes, mesenteric lymphnodes, as well as cells from the peritoneal cavity and Peyer's patches. Furthermore, we used CD19-sorted B cells for functional survival and proliferation assays.
Results: Akt^{BOE} mice displayed a spleenomegaly accompanied by enlarged lymph nodes compared to control mice. FACS analysis of bone marrow showed a reduction of re-circulating B cells while immature B cells were not affected. In the spleen we found a loss of CD23 expression by B cells. Further, we found increased absolute numbers of B and T cells and more neutrophiles, monocytes and mature macrophages in the spleen. Finally, the investigation of immunoglobulin titers by ELISA revealed reduced IgM, IgG1 and IgG3 serum levels.
Conclusions: The B cell specific overexpression of Akt underlines its importance in B cell maturation and homeostasis.CD23 serves as a

lymphoma-marker because of its differential expression on various types of lymphoma. The fact that Akt^{BOE} mice have almost no CD23 expression on B cells hinds towards some kind of lymphoma in these mice.

P0468

Ro52- and Ro60-specific B cell pattern in the salivary glands of patients with primary Sjögren's syndrome

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Purpose/Objective: Primary Sjögren's syndrome (pSS) is characterised by the presence of autoantibodies against the ribonucleoprotein (RNP) particles Ro/SSA and La/SS-B, and mononuclear cell infiltration of exocrine tissues, especially salivary and lachrymal glands. Low numbers of autoantigen-specific memory B cells and elevated levels of plasma cells have previously been detected in the peripheral blood (PB) of pSS patients compared to controls[1)]. Since both Ro52 and Ro60-specific cells have been detected in the salivary glands (SG) of pSS patients, we aimed to characterise the SSA-specific B cell pattern in SG biopsies.
Materials and methods: A series of double immunohistochemical stainings were performed on paraffin-embedded tissue from 10 well-characterised pSS patients for each Ro52 and Ro60 along with CD19, CD20, CD27, or CD5, respectively.
Results: Ro52 and Ro60-specific cells detected in SG tissue were found to be CD19+ B cells located outside the CD19+/CD20+ B cell zones (BCZ) and also interstitially. These SSA-specific cells were also quantified. No SSA-specific cells were observed within the CD20+ BCZ. Hence, no SSA-specific memory B cells were detected in these individuals. Contrary to this, SSA-specific cells were found to be CD19+/CD27++, demonstrating that they are differentiating short or long-lived plasma cells. Also, no SSA-specific cells were CD5+, indicating that they do not belong to the B-1 B cell subset.
Conclusions: Together our findings suggest that these lower levels of SSA-specific memory B cells in PB and absence of SSA-specific memory B cells in SG of pSS patients could be resulting from activation of these cells into plasma cells at the site of inflammation.

1. Aqrawi LA, Skarstein K, Bredholt G, Brun JG, Brokstad KA. Autoantigen-specific memory B cells in primary Sjogren's syndrome. *Scand J Immunol* 2012;75(1):61−8.

P0470

Sustained B-cell receptor signaling prevents B cell terminal differentiation into plasma cells

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Purpose/Objective: B-cell terminal differentiation into antibody secreting plasma cells (PC) features a transcriptional shift driven by the activation of plasma cell lineage determinants. Little is known about the signals inducing this change in transcriptional networks and the role of the B Cell Receptor (BCR) in terminal differentiation remains especially controversial.
Materials and methods: We used immunoglobulin light chain transgenic mice expressing suboptimal surface BCR levels and LMP2A knock-in animals with defined BCR-like signal strengths to explore the influence of BCR signaling in terminal differentiation independently of cognate antigens.
Results: We observed a strong increase of PC numbers both *in vivo* and *in vitro* upon weak, antigen-independent constitutive BCR signaling. The PI3K/Akt pathway appears to be involved in this exacerbated terminal differentiation. Interestingly, in our models, terminal differentiation occurs independently of antigen trigerring, germinal center formation or T-cell help yet giving rise to long-lived PC in spleen. In striking contrast, strong constitutive BCR signaling, mimicking chronic contact with cognate antigen, completely prevented both *in vitro* and *in vivo* PC differentiation.

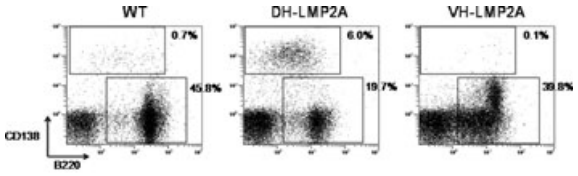

Figure 1. Plasma cell development in BCR-negative LMP2A mice models. DH-LMP2A mice display a weak BCR-like signal while VH-LMP2A display a strong constitutive signal. Spleen cells from 8 weeks old mice were analysed.

Conclusions: We demonstrated an inverse correlation between BCR signal strength and PC development. These findings provide new insights onto the role of the BCR in PC differentiation and point to the need to resolve BCR signaling to guarantee terminal differentiation.

P0471

Syk is required in B cells for effective antibody responses

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Purpose/Objective: Spleen tyrosine kinase (Syk), a pivotal kinase in B cell signalling, is recruited to phosphorylated tyrosines in immunoreceptor tyrosine-based activation motifs (ITAMs) after stimulation of the B cell receptor. Binding to these motifs leads to phosphorylation of Syk and subsequent activation of important downstream signalling molecules. Syk-deficient mice have no mature B cells due to a complete block in B cell development at the immature stage. To study immune responses of Syk-deficient primary B cells, we used a mouse model, which allows inducible deletion of Syk.
Materials and methods: To achieve inducible deletion of Syk in mature B cells, we used mice, carrying Cre recombinase fused to two mutated oestrogen receptors and a floxed allele of the S*yk* gene together with either a wildtyp or a knockout allele of the *syk* gene. These mice were treated with tamoxifen to induce Syk deletion. Immune responses and B cell activation in these mice were analysed *in vivo* and *in vitro*.
Results: B cell numbers drop after inducible Syk deletion, but a significant proportion of Syk-deficient B cells survives on a long time scale. We showed that Syk-defcient B cells are unresponsive to BCR stimulation and less responsive to stimulation of TLR recptors. Mice with Syk-deficient B cells had reduced titers of immunoglobulin after immunization with thymus-dependent or thymus-independent antigens. In addition mice with Syk-deficient B cells show strongly impaired germinal centre formation.
Conclusions: A long-lived B cell population remains after deletion of Syk. These cells appear to be impaired in responding to BCR and TLR stimulation and have impaired abilities to form germinal centre B cells.

P0472

The deubiquitinases A20 and CYLD do not share overlapping functions during B cell differentiation and activation

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Purpose/Objective: The deubiquitinases TNFAIP3/A20 and CYLD are critical negative regulators of signaling events leading to the activation of NF-κB transcription factors. They share similar mechanisms by removing non-degradative K63-linked polyubiquitin chains from an overlapping set of substrates. Loss of A20 in B cells results in impaired immune homeostasis, chronic inflammation and autoimmunity. In contrast, the consequences of CYLD-deficiency in B cells are controversial, ranging from an absence of effects to dramatic B cell hyperplasia. These differences could be due to varying compensation by A20. The aim of this study is to address a potential redundancy between A20 and CYLD in B cells.

Materials and methods: We generated mice lacking both A20 and CYLD in B cells and studied B cell differentiation in these mice. In addition, we investigated the responses of the A20/CYLD-deficient B cells to different B cell mitogens by measuring the activation status, proliferation and cytokine production *in vitro*.

Results: The combined loss of A20 and CYLD did not exacerbate the developmental defects of A20-deficiency in B cells. In addition, loss of both A20 and CYLD did not have additive effects on B cell activation, proliferation and the NF-κB-dependant production of the proinflammatory cytokine IL-6.

Conclusions: We concluded from this study that the lack of phenotypic effects in CYLD-deficient mice is not due to compensation by A20 but that A20 and CYLD do not functionally cooperate during B cell differentiation and activation.

P0473

The *in vivo* role of Blimp-1 SUMOylation in B Cell Development

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Purpose/Objective: Recent *in vitro* studies have shown the importance of SUMOylation (a ubiquitin-like posttranslational modification) on lymphocyte development, in particular early lymphocyte and Plasma cell (PC) development. PC development and differentiation is controlled by BLIMP-1, a transcriptional repressor that is necessary and sufficient to allow B cells to differentiate into antibody secreting cells. The study presented here uses a B cell specific Sentrin protease 1 (SENP1, a deSUMOylation protease) conditional knockout mouse model to investigate the influence of SUMOylation on lymphocyte development, mainly focusing on BLIMP-1 dependent PC development.

Materials and methods: The role of BLIMP-1 SUMOylation was investigated in SENP1 conditional knockout mice crossed to CD19-Cre and AID-Cre lines, therefore lacking SENP1 from early B cell developmental stage and following the germinal center reaction, respectively. Different B cell subsets were analyzed by flow cytometry, quantitative real time-PCR and Western blot. Sheep red blood cells were used for *in vivo* stimulation whereas Lipopolysaccharide and IL-4 was used for *ex vivo* stimulation.

Results: We discovered that BLIMP-1 undergoes a reversible modification with SUMO-1, which facilitates BLIMP-1 turnover and proteasomal degradation. Increase in SENP1 activity stabilized BLIMP-1, while a decrease promoted its degradation. This data indicates that SUMOylation of BLIMP-1 regulates its intracellular stability. In *in vivo* experiments we can show an impairment of B cell development due to a decreased amount of total B cells in these mice. Furthermore, in naïve mice, we observe increased numbers of immature B cells in spleen and lymph nodes and of germinal center B cells in Peyer's patches, as well as in spleens of sheep red blood cell immunized mice. We could also show *ex vivo* an impaired ability of B cell survival after Lipopolysaccharide (LPS) and IL-4 stimulation.

Conclusions: This study shows a direct influence of posttranslational SUMOylation on B cell development in an *in vivo* mouse model. In our preliminary data the elevated germinal center B cell numbers underline the influence of SUMOylation on BLIMP-1 activity, as impaired SUMOylation of BLIMP-1 leads to its degradation and therefore an absence of termination of the germinal center reaction.

P0474

The kinetics of a memory B cell response to virus like particles in mice

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Purpose/Objective: Vaccine-induced specific antibodies are usually responsible for protection against infection. The most common protective mechanism of antibodies is binding to pathogens which neutralizes their infectious potential and enhances their elimination by phagocytes. The most potent antibodies are produced by B cells which have undergone a germinal centre reaction. Within germinal centres, the BCRs of the specific B cells switch from IgM to IgG and hypermutate by the insertion of point mutations in their variable regions. Upon hypermutation, B cells are selected through iterative cycles of best fit for the antigen by follicular dendritic cells (FDC) and follicular T helper cells. While the GC reaction is usually relatively short-lived and ends a few months after elimination of the pathogen, memory B cells persist in the host for very long time-periods and may rapidly respond upon re-encounter of the antigen. In contrast to the well understood differentiation of naïve B cells into memory and/or plasma cells, relatively little is known about the fate of memory B cells upon re-exposure to antigen. Since classical vaccines often only induce protective antibodies after several injections, it is important to understand how triggering of memory B cells may affect the development of high affinity and long lasting antibody responses.

In this study we followed the kinetics of the specific memory B cell response in terms of antibody production and frequencies of specific B cells.

Materials and methods: To address this question, we used virus-like particles derived from the bacteriophage Qβ, which are highly immunogenic due to their repetitive structure. To trace memory B cells, congenic Ly5.1+ C57BL/6 mice were immunized with Qβ, splenic memory B cells were isolated and transferred intravenously into naïve Ly5.2+ C57BL/6. Twenty-four hours later recipient mice were challenged with Qβ.

Results: Transferred memory B cells homed the spleen. Although they did not proliferate as much as naïve B cells after immunization they differentiated more quickly into plasma cells and secreted more antibodies. The number of plasma cells found in spleen is similar in a primary and memory response. However, a higher number of plasma cells were found in the bone marrow, the main source of humoral immunity, after transfer of memory B cells.

Conclusions: Memory B cells respond to antigen by rapid differentiation into plasma cells rather than extensive proliferation. The induced plasma cells preferentially home to the bone marrow and produce larger amounts of antibodies than plasma cells derived from naïve cells.

P0475
The lymphoid/myeloid potential of B-1 cells is sustained by WNT/beta catenin pathway

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Purpose/Objective: The Wnt/beta-catenin signaling pathway has been shown to play an important role in controlling the proliferation, survival, and differentiation of hematopoietic cells. Several Wnt/β-catenin signaling components substantially influence hematopoietic cell fate. We have shown that B-1 cells are self-renewing cells and spontaneously express both myeloid and lymphoid restricted transcription factors. B-1 lymphocytes play a major role in autoimmunity and are related to CD5+ B-cell lymphomas and leukemias, such as CLL (chronic lymphatic leukemia). Previously, we have shown that this pathway influences B-1 cell proliferation *in vitro*. Considering that Wnt/beta-catenin signaling network as a critically important regulator of hematopoiesis, we have investigated the effect of quercetin, a classical Wnt inhibitor, on B-1 cells hematopoietic lineage-restricted genes expression.
Materials and methods: Peritoneal B-1 cells were obtained and cultivated as described by Popi *et al.* (*Immunology* 2009;126(1): 114–22). Treatment of B-1 cells with the Wnt inhibitor quercetin induced an inactivation of the Wnt pathway and suppressed cell proliferation. Herein we have analyzed the effect of Wnt inhibition on the expression of lymphoid and myeloid genes by B-1 cells by real time PCR.
Results: We have demonstrated that Wnt/beta-catenin signaling is inhibited by quercetin treatment of B-1 cells *in vitro*. Wnt pathway inhibition by quercetin induces silencing in Pax-5 and CD19 expression by B-1 cells. However, expression of myeloid associated genes by B-1 cells is still observed after quercetin treatment.
Conclusions: Inhibition of Wnt pathway interferes with the commitment of B-1 cells to B-cell lineage, suggesting that Wnt pathway plays an important role during B-1 cell differentiation. Considering that quercetin could inhibit leukemic cell growth without suppressing normal hematopoiesis, the role of Wnt pathway on B-1 cell proliferation and differentiation could be closely related to their ability to generated leukemic cells. Based on this, B-1 cells could be considered an *in vitro* model to study the molecular networks that orchestrate the transformation of cancer stem cell.

P0476
The role of B lymphocytes in alpha-1-antitrypsin mediated allogeneic allograft protection

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Purpose/Objective: Type-1 diabetes (T1D) is characterized by immune-mediated islet beta cell destruction. Islet transplantation restores normoglycemia and is considered for clinical implementation in T1D patients. There is evidence for B lymphocyte involvement in T1D as well as in islet allograft rejection. However, a protective role for B cells is suggested by the occurrence of islet allograft rejection in B cell-knockout mice. Alpha-1-antitrypsin (AAT) is an anti-inflammatory circulating protein that promotes tolerance towards islet allografts and prevents diabetes in non-obese diabetic (NOD) mice in a yet unknown mechanism. Aim: To characterize the effect of AAT on B lymphocyte responses in the context of allogeneic islet transplantation.
Materials and methods: Spleen-derived B lymphocytes were studied. B cell activation-related responses and expression of B cell activating factor (BAFF) receptor were determined under stimulatory conditions in the presence or absence of human AAT (hAAT, 0.5 mg/ml). *In vivo,* B cell proliferation response evoked by allogeneic skin transplantation was evaluated in draining lymph nodes (DLN) in transgenic mice that express constitutive hAAT ($hAAT^{+/+}$). To determine whether the allo-protective role of hAAT is B cell-dependent, B cell-depleted $hAAT^{+/+}$ chimeras were generated; the mice were rendered diabetic by streptozotocin and then transplanted with allogeneic islets.
Results: In the presence of hAAT, B cells exhibited a decline in CD40 ligand-induced co-stimulatory molecules CD86 and CD80 (6.3 ± 0.1% and 33.7 ± 4.1%, respectively; $P < 0.05$). B cells stimulated with anti-IgM $(Fab)_2$ displayed 35 ± 5.2% less surface BAFF receptor in the presence of hAAT. In cultures stimulated with CD40 ligand, LPS or BAFF, hAAT diminished inducible B cell proliferation 2.79 ± 0.13-fold, 4.62 ± 0.63-fold and 2.04 ± 0.21-fold, respectively ($P < 0.05$). Skin allograft-evoked B cell proliferation was 4.36 ± 1.1-fold lower in $hAAT^{+/+}$ mice than in WT recipients ($P = 0.0398$). While $hAAT^{+/+}$ transgenic mice accepted islet allografts, chimeric $hAAT^{+/+}$ mice (<7% circulating B cells) rejected allografts by 30 days after transplantation.
Conclusions: Our results suggest that hAAT modulates B cell responses in a manner that benefits islet allograft survival, and its protective properties are B cell-dependent.

P0477
The role of the deubiquitinating enzymes CYLD and A20 in B-cell maintenance and maturation

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Purpose/Objective: The NFκB-pathway plays a central role in the mammalian immune system regulating processes, ranging from development and survival of the lymphocytes to controlling the immune response to invading pathogens. In addition it was shown that it is a mechanistic link between inflammation and cancer and therefore its regulation is extremely important. CYLD and A20 are both negative regulators of the NFκB pathway by removing activating lysine-63 linked ubiquitin chains from numerous NFκB signaling molecules. Furthermore A20 is able to target those for proteasomal degradation by editing lysine-48 linked ubiquitin chains. Our aim was to shed light upon the role of the deubiquitinating enzymes CYLD and A20 in B cell maintenance, maturation, controlling the NFκB-pathway and cancer development.
Materials and methods: We crossed $CYLD^{Fl/Fl}$ mice to mice that lack A20 expression in B cells ($A20^{Fl/Fl}$CD19Cre), thereby creating a mouse strain deficient for both FL-CYLD, and A20 in B cells. These B cells over-express sCYLD, a naturally occurring splice variant of FL-CYLD, lacking the binding domains for TRAF2 and NEMO.
Results: These mice exhibit an expansion of the B cell compartment in the peripheral lymphatic organs as well as an increase in germinal center B cell formation. B cells isolated from these mice show prolonged survival and increased proliferation after α-IgM and CpG stimulation compared to CD19Cre control mice. In contrast to the B cell specific knockout of A20, which showed a significant reduction of marginal zone cells and a shift to the B2 B cell compartment in the peritoneal cavity, B cell specific double KO has an increased marginal zone B cell population and an increased B1a B cell population in the peritoneal cavity. Interestingly, these double KO mice exhibit a significantly elevated B1a cell population

in all secondary lymphatic organs, together with a three-fold increase of CD5 expression on B cells in the blood.

Conclusions: These data indicate a role of CYLD or respectively sCYLD downstream of A20. In addition the abundant appearance of B1 cells in lymphatic organs coupled with an increased expression of CD5 and CD23 on B cells, enhanced B cell receptor (BCR) signaling and survival might point to chronic lymphocytic leukemia (CLL) development.

P0478

The role of the inhibitory receptor SiglecG in the development of autoimmunity

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Purpose/Objective: Siglec-G is a member of the Siglec (sialic acid-binding immunglobulin-like lectin) family and acts as an inhibitory receptor of B1 cells. Siglec-G deficient mice show an expanded population of B1 cells, which have a higher calcium response to B-cell receptor signalling. Furthermore, higher serum IgM titers were observed. However, Siglec-G deficient mice in the BALB/c background did not develop spontaneous autoimmunity, which led us to further characterise the potential role of SiglecG in autoimmune models.

Materials and methods: SiglecG deficient mice in the BALB/c background were challenged with chicken type II collagen to induce rheumatoid arthritis. The SiglecG deficient mice were backcrossed into the SLE prone and fas-deficient MRL/MpJ fas/lpr background using speed congenics.

Results: We demonstrate that BALB/c Siglec-G deficient mice challenged with collagen-induced arthritis showed a more severe course of disease in terms of incidence and clinical score of arthritis signs than wildtype mice. These results were confirmed via H&E staining on knee sections.

Five month old Siglec-G-deficient MRL/MpJ fas/lpr mice show no difference in average life span compared to wildtyp MRL/MpJ fas/lpr mice but the incidence and severity of arthritis was more pronounced in Siglec-G deficient MRL/MpJ fas/lpr mice. Female Siglec-G knockout MRL/MpJ fas/lpr mice had an earlier onset of proteinuria (>300 mg/dl). Furthermore we observed a higher titer of anti-dsDNA IgG antibodies and rheumatoid factors in Siglec-G-deficient MRL/MpJ fas/lpr mice.

Conclusions: Even though SiglecG deficient mice in the BALB/c background do not show spontaneous autoimmunity, based on our data we conclude that the loss of the inhibitory receptor SiglecG can contribute to the development of autoimmunity, both in mouse SLE and rheumatoid arthritis models.

P0480

The role of toll-like receptor 9 in B-cell activating factor (BAFF) expression and function in normal human B-cells

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Purpose/Objective: B-cell autoreactivity is a characteristic abnormality in several autoimmune diseases. The inappropriate activation of toll-like receptors (TLRs) and/or the over-expression of B-cell activating factor (BAFF) have increasing importance in breaching B-cells' self-tolerance. In systemic lupus erythematosus (SLE), activation of TLR7 and TLR9, accompanied by high serum levels of BAFF, are implicated in disease pathogenesis. In murine B-cells, TLR9 activation resulted in up-regulating BAFF expression, while such a direct effect has not yet been established in normal human B-cells (nhB-cells). We therefore studied the effect of CpG-2006, a synthetic TLR9 ligand, on the expression of BAFF and its receptors (BAFF-R, TACI and BCMA) by nhB-cells.

Materials and methods: qPCR, flow cytometry and ELISA assays were utilized to investigate the effect of 3μg/ml CpG-2006 on BAFF expression at the level of mRNA, intracellular and membrane-bound protein expression, and secreted protein expression, respectively. BAFF receptors expression, in response to 3 μg/ml CpG-2006 was explored using flow cytometry. The functional role of membrane-bound BAFF was studied in BCR co-simulation and blocking assays.

Results: BAFF expression, in response to CpG-2006, was significantly upregulated at the level of mRNA, intracellular and membrane-bound protein. In contrast, we did not detect secreted BAFF in culture supernatants of CpG-2006-stimulated nhB-cells. CpG-2006, in addition, promoted the expression of TACI receptors, but not of BAFF-R or BCMA, in nhB-cells. We found that CpG-stimulated B-cells co-stimulated B-cell receptor-induced cellular proliferation of nhB-cells, an effect that was completely blocked by a BAFF-specific monoclonal antibody. Finally, CpG-2006 treatment of nhB-cells sensitised them to proliferate in response to exogenous BAFF, whereas exogenous BAFF had no effect on the proliferation of untreated B-cells. This effect was not mediated through TACI, as a TACI-specific blocking antibody failed to inhibit BAFF-mediated cellular proliferation.

Conclusions: Taken together, these novel findings demonstrate a functional cross-talk between TLR9 and BAFF signaling in nhB-cell, resulting in autocrine B-cell activation, and have implications for the roles of TLR9 and BAFF in the pathogenesis of SLE.

P0481

TLR9, CD40 or BCR stimulated human naïve and memory B cells exhibit differential responsiveness to the survival effect of IL-21

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Purpose/Objective: IL-21 is one of the most potent cytokines for human B cell proliferation and differentiation. IL-21 also influences B cell survival. The stimulatory or inhibitory effect of IL-21 depends on the maturation and activation status of the B cell, the co-stimulatory accompanying signals and the presence of other cytokines. The aim of this study was to evaluate the response of human naïve and memory B cells to IL-21 after TLR9, CD40 or BCR engagement.

Materials and methods: B cells were obtained from PBMC by negative selection using magnetic beads. Purified CFSE-free or CFSE-labelled B cells were cultured 3 days in the presence of ODN, anti-CD40 antibody or anti-IgM alone or in combination with human recombinant IL-21. Collected cells were stained with anti-CD19PCy7 and anti-CD27PCy5 monoclonal antibodies and analyzed by flow cytometry. Annexin V-FITC and Propidium Iodide staining protocol was used to evaluate the apoptosis of CSFE-free naïve ($CD19^+$ $CD27^-$) and memory ($CD19^+$ $CD27^+$) B cells. Proliferation was measured by CSFE dilution in previously gated CSFE-labelled cells.

Results: Spontaneous apoptosis was higher in memory than in naïve B cells. All stimuli alone protected both B cell subsets from spontaneous apoptosis. When IL-21 was tested alone, only naïve B cells were rescued from apoptosis. In contrast, IL-21 addition reverted the protective effect of all other stimuli on naïve B cells. In memory B cells, IL-21 was also able to revert the protective effect of anti-IgM and CpG-ODN but not anti-CD40.

Anti-IgM, anti-CD40, or IL-21 alone did not induce proliferation of naïve or memory B cells. CpG-ODN alone induced proliferation only on memory B cells. The combination of IL-21 with anti-IgM did not induce proliferation. IL-21 and anti-CD40 induced greater proliferation of memory than naïve B cells. The addition of IL-21 decreased

CpG-ODN induced proliferation of memory B cells and modestly increased proliferation of naïve B cells.
Conclusions: Memory B cells are more sensitive to spontaneous apoptosis than naïve B cells and less prone to be rescued by individual stimuli. Although a survival factor for unstimulated naïve B cells, IL-21 abrogates activation-induced survival of naïve B cells. On the contrary, IL-21 selectively supports memory B cell responses to T dependent stimulus.

P0482

WASp and N-WASp regulate the germinal center response and production of auto-antibodies

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Purpose/Objective: Wiskott-Aldrich syndrome (WAS) is a rare, potentially life threatening X-linked primary immunodeficiency disease. Forty to 70% of the patients develop any form of autoimmunity. WAS is caused by the WAS protein (WASp). We hypothesized that mice lacking WASp family members have a skewed development and activation of B cells due to intrinsic B cell dysfunction and decreased protective shield of the splenic marginal zone to blood-borne antigens.
Materials and methods: To determine the role of WASp and the homologues molecule N-WASp in B cell biology we have used WASp knock out (WKO) mice and mice lacking WASp and N-WASp in B cells (cDKO mice). To induce breakdown of tolerance with emergence of auto-antibodies, we immunized mice with apoptotic cells.
Results: Compared to reduced uptake and decreased immune response after non-self antigen immunization, WKO and cDKO mice had normal uptake of apoptotic cells in the marginal zone and formed large germinal centers after apoptotic cell immunization. However, compared to germinal center B cells in wild type mice, B cells retained longer and proliferated less in germinal centers of WKO and cDKO mice, suggesting decreased capacity to undergo affinity maturation. When compared to wild type mice, WKO and cDKO mice had significantly higher DNA-specific IgM antibodies before and after immunization with apoptotic cells. After repeated apoptotic cell immunizations the immunological tolerance was broken in wild type mice that produced DNA-specific IgG antibodies, while WKO and cDKO mice failed to produce anti-DNA IgG antibodies.
Conclusions: Together, our data show that mice lacking WASp family members respond with an atypical immune response to self-antigens such as apoptotic cells. WKO and cDKO B cells formed low quality GC-like structures upon self-antigen challenge and produced mainly auto-reactive IgM antibodies. Our data will increase the knowledge of why patients with primary immunodeficienies such as WAS develop autoimmune diseases.

Poster Session: Cytolytic T Cells

P0483

An optimal pMHCI/CD8 interaction exists which affords maximum pMHCI sensitivity without loss of CD8$^+$ T-cell specificity

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Purpose/Objective: CD8$^+$ T-cells recognize 'foreign' peptide fragments, in the context of 'self' major histocompatibility complex class I (MHCI) molecules on the surface of host cells. The detection of T-cell antigens is unique because it involves the binding of two receptors (TCR and CD8) to a single ligand (pMHCI). The pMHCI/CD8 interaction is characterized by very low solution binding affinities (K_D = 137 μM). An incremental enhancement in CD8 binding (~1.5-fold) has previously been shown to result in enhanced recognition of pMHCI, thereby demonstrating the huge potential of CD8 in strategies to enhance T-cell immunity. I aim to define the strength of the pMHCI/CD8 interaction which affords maximal enhancement of pMHCI recognition whilst still retaining specificity of pMHCI recognition by the TCR.

Materials and methods: In this study, I used a system of MHCI mutants which vary in the strength of the pMHCI/CD8 interaction. Using tetramer technology, I constructed a panel of A*0201 pMHCI tetramers that exhibit an abrogated (DT227/8KA), weak (A245V), normal (wild-type), slightly enhanced (Q115E), intermediately enhanced (A245V/K^b) and super-enhanced (K^b) interaction with CD8. The latter two mutations were also combined with a reduced affinity β2-microglobulin mutation (β2M K58E). This tetramer panel was then used to examine how the strength of the pMHCI/CD8 interaction influences pMHCI recognition at the cell surface and the specificity of pMHCI recognition.

Results: TCR recognition of pMHCI at the cell surface increases as the strength of the pMHCI/CD8 interaction is increased. Loss of pMHCI tetramer staining specificity was observed at pMHCI/CD8 strengths exceeding a threshold K_D of ~30 μM. Thus, an optimal pMHCI/CD8 binding interaction exists (between a K_D of 97 and 30 μM) which increases pMHCI sensitivity without the loss of specificity.

Conclusions: This data defines the strength of the pMHCI/CD8 interaction which affords maximal enhancement of pMHCI recognition whilst still retaining specificity of pMHCI recognition. Due to the non-polymorphic nature of CD8, strategies to enhance the antigen specific T-cell response by targeting CD8 would be globally applicable to any system in which enhanced T-cell immunity is desirable.

P0485

CD40L induced by strong cytotoxic T cell epitopes on CD8 T cells contributes to helper-independent responses

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Purpose/Objective: CD8 T-cell responses can be primed by strong cytotoxic T cell epitopes even in the absence of CD4 epitopes or DC-activating adjuvants. The aim of this work is to analyze the role of CD40/CD40L interaction on the induction of helper-free CD8 T cell responses.

Materials and methods: Different strains of mice were immunized with different cytototoxic T cell epitopes emulsified in incomplete Freund adjuvant. CD40/CD40L interaction was blocked *in vivo* and *in vitro* by anti-CD40L antibodies. DC maturation as well as IFN-gamma/CD40L expression by primed CD8 T cells was measured by flow cytometry. Also, IFN-gamma production was analyzed by ELISA and ELISPOT.

Results: We found that CD8 T-cell responses induced by immunization with strong cytotoxic T cell epitopes were inhibited *in vivo* by CD40L blockade. *In vitro*, peptide stimulation of splenocytes from immune mice induced CD40L on CD8 T cells and DC maturation, which was partially inhibited by anti-CD40L antibodies. Interestingly, CD40L blockade also inhibited CD8 responses, even in the presence of already mature DC, suggesting a role for CD40L not only in DC maturation but also in CD8 costimulation. These peptides had features of CD4 epitopes, since they helped the induction of responses against other less immunogenic CD8 epitopes. Finally, analysis of peptide epitopes used in human vaccination clinical trials showed that they also induced CD40L on CD8 cells.

Conclusions: These results suggest that CD40L expression induced by strong CD8 peptide epitopes can facilitate activation of helper-free CD8 responses.

P0486

CD8beta ADP-ribosylation regulates CD8 coreceptor function

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Purpose/Objective: The coreceptor of conventional CD8$^+$ T cells consists of a CD8ab heterodimer and correct interaction of CD8a, CD8b, TCR, and MHC-I is decisive for antigen recognition and effective signal transduction. ADP-ribosyltransferase 2 (ART2) is an ectoenzyme expressed on T cells that utilizes extracellular nicotinamide adenine dinucleotide (NAD) to transfer ADP-ribose to arginine residues of membrane proteins. Here we show that CD8b on murine CD8$^+$ T cells becomes ADP-ribosylated *in vitro* and *in vivo* in the presence of extracellular NAD.

Materials and methods: ADP-ribosylation leads to loss of binding of certain anti-CD8b antibodies (YTS156.7.7 and 53−5.8). Other anti-CD8b antibodies (H35-17) still recognize CD8b indicating that NAD causes only modification of certain epitopes and not a general loss of CD8b.

Results: The ADP-ribosylation of CD8b is mediated by ART2, because loss of ART2 expression results in lack of CD8b ADP-ribosylation even in the presence of high doses of NAD. Furthermore, loss of ADP-ribosyl cyclase 1 (CD38), an ectoenzyme that degrades extracellular NAD, results in inevitable CD8b ADP-ribosylation upon any cell isolation procedure. ADP-ribosylation of CD8b is stable for several hours and re-appearance of unmodified CD8b is mainly due to the replacement of ADP-ribosylated surface CD8b by newly generated molecules, but there also appear to be mechanisms that remove ADP-ribose residues from surface CD8b. Finally, NAD treatment of ovalbumin-specific endogenous CD8$^+$ T cells or of T cells from OT1 transgenic mice substantially reduces binding of ovalbumin-MHC-I tetramers. Since activated CD8$^+$ T cells downmodulate ART2 expression, reduction of tetramer binding is less pronounced on effector CD8$^+$ T cells. Nevertheless, an *in vivo* cytotoxicity assay revealed impaired CD8$^+$ T cells function after i.v. NAD injection.

Conclusions: In summary, we propose that ADP-ribosylation of CD8b can regulate the coreceptor function of CD8 on CD8$^+$ T cells in the presence of elevated levels of extracellular NAD. Our current studies aim at elucidating the role of this mechanism for the regulation of CD8$^+$ T cell responses.

P0487

Cross reactivity prediction based on hierarchical clustering of pMHC complexes

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Purpose/Objective: Cross-reactivity was initially described as being triggered by the great similarity between the amino acid sequences of virus-derived peptides presented in the context of the Major Histocompatibility Complex (MHC). These peptide:MHC (pMHC) complexes are recognized by Cytotoxic T Lymphocytes (CTLs), which are the effector agents of cellular immunity. However, immunologists have already described many events of cross-reactivity between epitopes sharing <50% of the linear amino acid sequence, and this phenomenon has proven to be far more difficult to predict.

Materials and methods: In the present work, we analyzed 60 unrelated pMHC complexes presenting virus-derived peptides, in the context of the most frequent human MHC allele (HLA-A*02:01). These complexes were obtained from the CrossTope Data Bank for Cross-Reactivity Assessment, being 5 crystal structures and 55 *in silico* predicted structures. Images of the TCR-interacting surface of these complexes, presenting the electrostatic potential distribution, were used to extract data from the points of interaction with the TCR. Values of these regions were used as input for multivariate statistical methods, aiming to predict possible targets of cross-reactivity. Aiming to compare our approach with other available strategies, we also used the same dataset as input to the *webPIPSA*.

Results: Our dataset included some peptides with already known cross-reactivity, and a Hierarchical Cluster Analysis (HCA) of these data agreed with this experimental background, presenting better results than the *webPIPSA* server. For instance, we included 10 variants of the wild-type epitope HCV-$NS3_{1073}$. All the variants with *in vitro* confirmed cross-reactivity were placed in the same group ('HCV-cluster'), while the noncross-reactive variant (from HCV genotype 3) was placed in a completely unrelated group. There is yet another complex included in the 'HCV-cluster', presenting the 'LLWTLVVLLO' peptide, from the Human herpesvirus 4 (LMP_{2329}). It is important to note that this peptide does not share even a single amino acid with the target peptide (CVNGVCWTV) and, nevertheless, presented almost the same structural pattern when presented in the context of HLA-A*02:01.

Conclusions: This result stresses the power of this structural approach in prospecting new cross-reactive targets.

P0488

Differential antigen-dependency of $CD4^+$ and $CD8^+$ T cells

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Purpose/Objective: T cell expansion and effector differentiation are triggered and, perhaps, maintained by antigen, but the proliferative behaviors of $CD4^+$ and $CD8^+$ T cells have rarely been compared side by side.

Materials and methods: TCR transgenic $CD4^+$ or $CD8^+$ T cells were stimulated for two days *in vitro* with CD3 and CD28 monoclonal antibodies and proliferation was followed upon transfer into mice expressing cognate antigen or not. CFSE dilution and cell cycle analysis were employed to characterize proliferative patterns of both subsets. To investigate the contribution of coinhibitory signaling and inflammatory stimuli, we employed blocking antibodies against coinhibitory molecules and bystander MCMV infection. Gene expression analysis was used to assess general differences between $CD4^+$ and $CD8^+$ T cells under conditions of transient or persistent TCR stimulation.

Results: We found $CD4^+$ T cell proliferation to be dependent on prolonged antigen presence, such that $CD4^+$ T cell proliferation ceased quickly after transfer into antigen-free mice, whereas $CD8^+$ T cells continued dividing. Furthermore, only $CD8^+$ T cells differentiated into effector cells and retained cell cycle activity following transient TCR stimulation. Gene expression analysis illustrated global differences in the transcriptomes of both subsets. We excluded $CD4^+$ T cell proliferation to be abrogated by coinhibitory signals or lacking inflammatory stimuli. Additionally, autonomous proliferation of $CD8^+$ T cells was independent of any MHC I signals. Our T cell data correlated with the decreased turnover of MHC II, but not MHC I, on activated dendritic cells *in vivo*.

Conclusions: Proliferation of $CD4^+$ and $CD8^+$ T cell subsets differentially dependent on antigen: $CD4^+$ T cells require persistent TCR stimulation for extended divisions and effector differentiation. However, a limited TCR stimulus is sufficient to drive proliferation and effector cell differentiation of $CD8^+$ T cells without maintenance of antigen presentation. This is reflected in the biology of MHC I, whose expression, unlike MHC II, is not stabilized on dendritic cells upon their activation *in vivo*.

P0489

in vivo elimination of drug-resistant tumor cells by cytotoxic T cells through a perforin/granzyme B-dependent mechanism

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Purpose/Objective: Cytotoxic T (Tc) and NK cells are key effectors in the control and/or elimination of tumor or virus-infected target cells. For this purpose they employ two distinct strategies, the engagement of death receptors and the granule secretory pathway, including perforin (perf) and granzymes (gzms). The main outcome of both processes is the induction of apoptosis of target cells. We have previously shown that *ex vivo*-derived perf and gzmB expressing virus-specific Tc cells are able to induce several distinct pro-apoptotic pathways *in vitro*.However, the relevance of these findings for the elimination of tumor cells *in vivo* is still not clear, a crucial question to predict the effectiveness of Tc cell-based immunotherapy protocols against cancer cells.

Materials and methods: By using virus-pulsed mice defective (knock out) in one or more components of the granule secretory pathway perf, gzmA, gzmB − we have now analysed the role of perf-dependent and -independent pathways in Tc cell-mediated elimination of transformed cells *in vivo*. To this aim, we have used fluorescence label tumor cells pulsed with virus antigens and analyse their specific elimination in the peritoneal cavity of primed mice.

Results: Efficient elimination of pulsed tumor cells by virus-specific Tc cells is critically dependent on perf. However, the relative contribution of the two major proteases, i.e. gzmA and gzmB, to tumor cell death was shown to depend on the amount of initial viral load. Thus, in mice infected with low viral doses (10 cfu), perf and gzmB are the major executors of tumor cell death, with gzmA only playing a minor role. Moreover, under these physiological conditions, perf and gzmB-expressing Tc cells are even able to eliminate multidrug resistant-tumor cells defective in the mitochondrial apoptotic pathway (i.e. overexpression of Bcl-X_L).

Conclusions: Our findings suggest that perf and gzmB-expressing Tc cells, previously educated to specifically recognise transformed cells are

a promising immunotherapeutic means to efficiently control tumor growth in patients refractory to conventional chemotherapies.

P0490

Interleukin-15 amplifies the pathogenic activity of $CD4^+$ CD28- T cells in multiple sclerosis patients

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Purpose/Objective: Immune ageing, or immunosenescence, contributes to the increased morbidity and mortality seen in the elderly. Premature immunosenescence is shown to occur in patients with multiple sclerosis (MS). The main characteristic of immunosenescence is the expansion of $CD4^+$ $CD28^-$ T cells in the peripheral blood. We showed that these cells accumulate in brain lesions of MS patients. $CD4^+$ $CD28^-$ T cells have a cytotoxic profile, shown by expression of the cytotoxic molecules perforin, granzyme B and NKG2D, an activating natural killer (NK) cell receptor. For NK cells, it is known that NKG2D expression and cytotoxicity increase in response to interleukin (IL)-15, which is upregulated in the serum and cerebrospinal fluid of MS patients. The aim of our study is to investigate whether IL-15 enhances cytotoxicity of $CD4^+$ $CD28^-$ T cells as seen for NK cells.
Materials and methods: Flow cytometric analysis was used to investigate proliferation, expression of cytotoxic molecules and degranulation of $CD4^+$ $CD28^-$ T cells in response to IL-15. Coculture with the NKG2D ligand expressing cell line U251 assessed the contribution of NKG2D ligation to degranulation of $CD4^+$ $CD28^-$ T cells. To identify IL-15 producing cells in the brain, immunohistochemistry was performed on MS lesion tissue and normal brain tissue of non-demented controls.
Results: We show that IL-15 preferentially induces proliferation of $CD4^+$ $CD28^-$ T cells as compared to their $CD28^+$ counterparts. Phenotypically, IL-15 significantly increases expression of NKG2D, perforin and granzyme B by $CD4^+$ $CD28^-$ T cells. Also, the production of interferon-gamma is increased, and this increase is significantly higher in MS patients. When IL-15 is presented to $CD4^+$ $CD28^-$ T cells, their release of cytotoxic granules is significantly enhanced. This degranulation is not dependent on NKG2D ligation, but blocking of NKG2D diminished this process. In MS lesions, we found that microglia/macrophages are the main IL-15 producing cells, and that $CD4^+$ T cells are found in close proximity to them, suggesting *in vivo* stimulation.
Conclusions: In summary, our findings indicate that $CD4^+$ $CD28^-$ T cells, which accumulate in brain lesions of MS patients, are functionally boosted by IL-15 producing cells. This process amplifies their cytotoxicity, contributing to local damage.

P0491

MAIT cells induce proinflammatory cytokine production in monocytes through the secretion of granzymes A and K

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Purpose/Objective: Mucosal-associated invariant T (MAIT) cells are a population of $CD161^{++}$ CD8 T-cells expressing the semi-invariant T-cell receptor iVα7.2, but their functions remain unclear.Here we examine the unique expression profile of cytolytic molecules in these cells. Granzyme (Gzm) A and Gzm K are trypsin-like serine proteases originally believed to function exclusively as pro-apoptotic proteases, but recent studies suggest that these molecules may possess proinflammatory functions.We therefore tested the hypothesis that granzyme release from MAIT cells induces proinflammatory cytokine production from target cells.
Materials and methods: Surface and intracellular staining followed by FACS analysis was performed using PBMCs isolated from 21 healthy donors, 12 chronically infected HIV patients and 13 chronically infected HCV patients. Enriched monocytes were treated with endotoxin removed Gzm A and Gzm K at 200nM (Enzo Life Sciences) before intracellular cytokine staining.Wilcoxon's matched pairs test was used for analyzing paired data, and the Mann-Whitney U test was used to compare unpaired data.
Results: We report that MAIT cells are characterized by a lack of Gzm B and perforin, but highly express Gzm A (99.5% $P < 0.0001$) and Gzm K (95.1% $P < 0.0001$) compared to CD161- CD8 T-cells. Furthermore, we found a dramatic increase of Gzm A ($P = 0.0007$) and Gzm B ($P < 0.001$) in total CD8 T-cells in HIV patients compared to healthy donors, while Gzm K was found to be reduced in HIV ($P = 0.0046$) and HCV patients ($P = 0.0282$). We show that extracellular Gzm K induces secretion of IL-1β in a time-dependent manner from monocytes. We also developed a novel FACS-based cytotoxicity assay to show that MAIT cells degranulate in an MR1-dependent manner in response to bacterially exposed APCs but they show low cytotoxicity to target cells.
Conclusions: MAIT cells express high levels of proinflammatory Gzms A and K but low levels of Gzm B and perforin. Triggering of these cells leads to degranulation but does not induce killing of target cells and induces secretion of IL-1β from monocytes.Together with their ability to secrete IL-17 and gut-liver homing characteristics, high expression of Gzm A and Gzm K most likely contributes to the antimicrobial and host defence properties of MAIT cells. Such activity may also play a role in tissue inflammation in diseases such as chronic HCV infection.

P0493

Monoclonal anti-CD8 therapy induces disease amelioration in collagen-induced arthritis in the B10.Q mouse model

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Purpose/Objective: $CD8^+$ T cells are known to infiltrate the synovium in rheumatoid arthritis (RA) in high numbers. However, their role in the pathogenesis of RA still needs to be fully understood. Using the collagen-induced arthritis (CIA) model in B10.Q mice, we aim at uncovering the potential of $CD8^+$ T cell depletion to stop the progression of the disease as well as its reversal.
Materials and methods: Arthritis was induced and scored in male 14–16 week-old B10.Q mice immunized with type II collagen emulsion. $CD8^+$ T cells from the peripheral blood were characterized for cell surface markers and intracellular cytokine production at three different time points: before CIA, at an intermediate arthritis level and at the peak of the disease, where they were then treated with a specific depleting anti-CD8 mAb (YTS169.4). Arthritic mice in the control group were treated with a mock antibody.
Results: The peripheral blood of B10.Q mice showed to decrease their $CD8^+$ T cell numbers in the periphery as the arthritis scores increased. It was also observed that the circulating $CD8^+$ T cells, despite diminishing in absolute numbers with the increase of arthritis scores, present a high percentage of the activation cell marker CD69, with the highest levels of expression observed at the peak of the disease. The intracellular production of granzyme B and proinflammatory cytokines such as TNFα and IFNγ by $CD8^+$ T cells also increased with arthritis scores.

Upon treatment with anti-CD8 mAb, the arthritis scores improved within 4 days of treatment, and remained consistently low for a period of 10 days. The control group maintained high arthritis scores. The resurgence of the $CD8^+$ T cells in the peripheral blood of $CD8^+$ T cell depleted mice is associated with the recovery of arthritis scores.

Conclusions: These results indicate that $CD8^+$ T cells have an activated phenotype in CIA in the B10.Q mouse model. The depletion of $CD8^+$ T cells from arthritic mice leads to an amelioration of the disease, which can indicate that $CD8^+$ T cells may have a greater role in the progression and maintenance of experimental arthritis.

P0495

System L amino acid transporter 1 (slc7a5) expression is crucial for T cell blastogenesis

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Purpose/Objective: Triggering of the T cell antigen receptor (TCR) causes T cells to up-regulate glucose, amino acid and iron uptake and switch to glycolysis. The objective of the present study was to identify the System L amino acid transporters that import large neutral amino acids (LNAA) such as leucine into T cells and then assess the importance of these transporters for T cell function. We now show that the System L amino acid transporter composed of the transporter light chain slc7a5, and heavy chain CD98 is expressed in immune activated but not naïve T cells. Moreover, the expression of this transporter complex is essential for T cell growth and proliferation.
Materials and methods: To address the importance of slc7a5 mediated amino acid transport in T cells, we have backcrossed mice expressing floxed slc7a5 alleles to mice expressing Cre recombinase under the control of the CD4 promoter. This drives T cell specific deletion of slc7a5, allowing analysis of the role for slc7a5 in peripheral T cells.
Results: CD4Cre $slc7a5^{fl/fl}$ mice have a normal peripheral T cell compartment indicating that this amino acid transporter is not required for T cell selection in the thymus or for the trafficking and homeostasis of the naïve T cell pool. However, $slc7a5^{-/-}$ T cells have a selective defect in T cell antigen receptor induced LNAA transport and fail to increase cell mass in response to immune activation. $slc7a5^{-/-}$ immune activated T cells cannot proliferate *in vitro* and *in vivo* and show defects in CD8 T cell differentiation to cytotoxic T cells. Moreover, $slc7a5^{-/-}$ CD4 T cells fail to differentiate to Th1 and Th17 although Treg differentiation is unaffected by lack of slc7a5. One striking observation was that $slc7a5^{-/-}$ T cells are unable to sustain expression of the glucose transporter Glut1 and hence have defective glucose uptake and cannot switch to a glycolytic metabolism. These studies afford the insight that the coordination of amino acid uptake and protein synthesis is essential for the glycolytic switch in T cells and for T cell activation.
Conclusions: The present data identify the regulated expression of System L amino acid transporter as a key event for T cell clonal expansion and demonstrate the importance of TCR regulated amino acid transport for peripheral T cell differentiation.

P0496

The effect of immunomodulation on IL-17^+ $CD8^+$ T cells in MS patients

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Purpose/Objective: In multiple sclerosis (MS), Th17 cells are believed to be pathogenic. We postulate that also IL-17^+ $CD8^+$ T cells are pathogenic since $CD8^+$ T cells are found in MS brain lesions and >70% of these cells are IL-17 positive. Therefore, we investigated the immune modulating effects of beta interferon (IFN-β), glatiramer acetate (GA) and vitamin D (VitD), on circulating $IL17^+$ $CD8^+$ T cells in MS.
Materials and methods: PBMC were isolated from healthy controls (HC; $n = 30$) and relapsing-remitting (RR) MS patients in remission (no medication: $n = 17$, IFN-β: $n = 18$, GA: $n = 13$) and during a relapse (no medication: $n = 12$, IFN-β: $n = 10$). Additionally, PBMC from 15 IFN-β treated RRMS patients, receiving 20 000 IU $VitD_3$/day for 12 weeks, were isolated at baseline and at 12 weeks. Intracellular FACS analysis was performed on PBMC to assess the IL-17^+ cell fraction in the $CD8^+$ T cell population. Data are given as median with corresponding interquartile range. Due to a correction for multiple testing, a P-value < 0.01 was considered significant.
Results: Compared to HC, MS patients in remission had higher IL-17^+ $CD8^+$ T cell percentages (0.3% [0.2−0.4] and 0.5% [0.3−0.5], respectively; $P = 0.001$), while MS patients during a relapse showed a trend towards higher percentages (0.5% [0.3−0.8]; $P = 0.016$). In patients in remission, the fraction IL-17^+ $CD8^+$ T cells did not differ between the three treatment groups. In MS patients during a relapse, IL-17^+ $CD8^+$ T cell percentages were similar in untreated and IFN-β treated patients (0.4% [0.2−1.4] and 0.7% [0.3−0.8], respectively). IL-17^+ $CD8^+$ T cell percentages were comparable before and 12 weeks after VitD supplementation (0.3% [0.2−0.5] and 0.4% [0.3−0.6], respectively).
Conclusions: These results show elevated IL-17^+ $CD8^+$ T cell percentages in MS patients. This, in combination with their presence in MS lesions, suggests a role for these cells in MS pathogenesis. However, therapy with either GA, IFN-β or vitamin D seemed unable to downregulate these cells in the circulation.

P0497

Viral dsRNA-activated human dendritic cells produce IL-27 which selectively promotes cytotoxicity in naïve CD8$^+$ T cells

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Purpose/Objective: Dendritic cells (DCs) are central in shaping immune responses by activating naïve T-cells, not only CD4$^+$ helper T cells but also CD8$^+$ cytotoxic T cells. Viral recognition programs DCs to express signal three molecules that promote the differentiation of effector CD8$^+$ T cells. Besides IL-12, another DC-derived IL-12-family member, IL-27, has been reported to contribute herein. Therefore, we studied the relative roles of IL-12 and IL-27 in the induction of CD8$^+$ T cell responses by human DCs.

Materials and methods: FACS sorted naïve CD8$^+$ T cells were activated either by anti-CD3/CD28 stimulation in the presence of recombinant human IL-12 and IL-27 or by viral dsRNA-activated human peripheral blood BDCA1$^+$ DCs in the presence of neutralizing antibodies against IL-12 or the IL-27 receptor. After 3–6 days CD8$^+$ T cell proliferation, granzyme B expression, cytokine production and cytotoxicity were determined.

Results: Whereas IL-12 potently induces inflammatory cytokines (i.e. IFN-g and TNF-a, but not IL-2), IL-27 excels in inducing proliferation and a cytotoxic profile (granzyme B, cytotoxicity of target cells) in human naïve CD8$^+$ T cells. Compared to bacterial cell wall peptidoglycan, viral dsRNA-mimic poly (I:C) is superior in priming human BDCA1$^+$ DCs to produce IL-12 and IL-27, which promote inflammatory cytokines and a cytotoxic profile in differentiating CD8$^+$ T cells, respectively.

Conclusions: This data supports the concept that viral dsRNA-activated human dendritic cells produce IL-27, which acts as a specialized pro-cytotoxic, anti-viral cytokine that promotes development of effector CD8$^+$ T cells.

Poster Session: Effector Th Cell Subsets and Plasticity

P0498
Activity of T helper cells in patients with primary Sjogren's syndrome

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Purpose/Objective: To investigate whether T helper (Th1, Th2 and Th17) cells activity can differentiate in peripheral blood of patients with primary Sjögren's syndrome (pSS), non- Sjögren's sicca syndrome (nSS-sicca) and healthy controls.

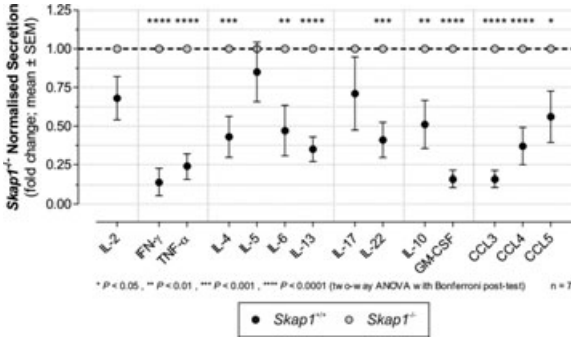
Materials and methods: We isolated peripheral blood mononuclear cells (PBMCs) from 34 pSS, 13 nSS-sicca patients and 13 healthy controls. We stimulated PBMCs using phorbol-12-myristate-13-acetate and ionomycin, labelled for CD4, IFN-γ, IL-4 and IL-17A and analyzed these cells using flow cytometry.

Results: According to our results no differences between nSS-sicca and healthy controls were found and we combined them in to one control group. Activity of Th1, Th2 and Th17 cells according to IFN-γ, IL-4 and IL-17A expression in patients with pSS were similar to control group. We found the significantly increased percentage of both IFN-γ and IL-17 producing Th17/Th1-like cells in pSS patients as compared to control group. We observed a significant correlation between all Th subsets activity in control group. Th1 correlated with Th17, with Th2 and Th17/Th1-like. Th2 correlated with Th17 and Th17/Th1-like. Th17 correlated with Th17/Th1-like. However, we observed correlation only between Th1 with Th2 and Th17 and Th17/Th1-like with Th17 in pSS group (see Table 1).

Table 1. Correlation of Th subsets in primary Sjögren's syndrome patients and control group

pSS group	Th subpopulation	Th1	Th17	Th2	Th17/Th1
	Th1	–	$r = 0.3654$ $P = 0.0261$	$r = 0.4657$ $P = 0.0048$	ns (not significant)
	Th17	$r = 0.3654$ $P = 0.0261$	–	ns	$r = 0.7485$ $P < 0.0001$
	Th2	$r = 0.4657$ $P = 0.0048$	ns	–	ns
	Th17/Th1	ns	$r = 0.7485$ $P < 0.0001$	ns	–
Control group	Th1	–	$r = 0.6811$ $P = 0.0001$	$r = 0.4997$ $P = 0.0093$	$r = 0.6184$ $P = 0.0008$
	Th17	$r = 0.6811$ $P = 0.0001$	–	$r = 0.3981$ $P = 0.0440$	$r = 0.7667$ $P < 0.0001$
	Th2	$r = 0.4997$ $P = 0.0093$	$r = 0.3981$ $P = 0.0440$	–	$r = 0.4925$ $P = 0.0106$
	Th17/Th1	$r = 0.6184$ $P = 0.0008$	$r = 0.7667$ $P < 0.0001$	$r = 0.4925$ $P = 0.0106$	–

Conclusions: The significantly increased percentage of Th cells producing both IFN-γ and IL-17A in peripheral blood suggest a possible role of Th17/Th1-like cells in the pathogenesis of pSS. We observed different correlation between Th subsets activity in pSS and in control groups. These results are especially important considering the fact that Th subsets activity levels seem to be unaltered in patients and controls. Therefore, we conclude that an imbalance of relationship between Th subsets activity plays a role in pSS pathogenesis.



P0499
Adaptor Src kinase-associated phosphoprotein-1 (SKAP1) differentially suppresses production of multiple cytokines and chemokines in $CD4^+$ T-cells

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Purpose/Objective: While immune cell adaptor Src kinase-associated phosphoprotein-1 (SKAP1, formerly known as SKAP-55) regulates integrin-mediated adhesion of T-cells, little is known whether it plays additional roles in modulating other aspects of immune responses. In this study, we report that while SKAP1 positively regulates T-cell adhesion, it elicited a paradoxical inhibitory effect on a selected group of cytokines and chemokines.

Materials and methods: Primary naïve *Skap1*$^{+/+}$ and *Skap1*$^{-/-}$ $CD4^+$ T-cells were stimulated by various concentrations of immobilized anti-CD3, anti-CD28 or unspecific IgG antibodies and the production of CCL3, CCL4, CCL5, CXCL10, GM-CSF, IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, IL-22, IL-27, TGF-β and TNF-α was simultaneously measured by FlowCytomix assay. In parallel, the dynamics of T-cell proliferation and cell viability was assessed by CFSE dilution and 7-AAD exclusion assays, respectively. The activity of mitochondrial oxidases was detected by MTT assays.

Results: SKAP1 suppressed the production of cytokines and chemokines such as IFN-γ, TNF-α, GM-CSF and CCL3 in the response to the T-cell receptor (TCR) stimulation. Th1 cytokines were affected more than Th2 cytokines, although the inhibition pattern did not strictly follow the division between Th1 and Th2 phenotype. SKAP1-dependent inhibition was not reversed by increasing the strength of the TCR signal, but unlike in the case of the other factors, IL-2 and CCL4 production could be restored by CD28 co-ligation. IL-17 production was relatively resistant to SKAP1-dependent inhibition. While this adaptor did not alter magnitude and dynamics of T-cell proliferation, it also limited the survival of non-dividing, but not proliferating $CD4^+$ T-cells.

Conclusions: SKAP1 represents the first example of an adaptor that can both enhance integrin-dependent T-cell adhesion and supress the production of certain cytokines and chemokines reducing polarization of the cytokine milieu towards Th1 phenotype.

P0500

Basophils control disease activity and T cell responses in experimental murine colitis

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Purpose/Objective: Basophils have been recognized as important inducers of T helper cell 2 (Th-2) responses in models of vaccination and infection. However, little is known about their role in autoimmunity. Using the colitis model of adoptive transfer of $CD4^+$ $CD62L^+$ T helper cells into lymphopenic hosts we analyzed whether basophils regulate T cell responses and modulate disease activity. We hypothesized that basophils are activated by proliferating T cells after transfer and influence colitogenic T cells.

Materials and methods: *In vivo* basophils were depleted with antibodies against FceR1 and CD2003R or expanded with repeated injections of recombinant interleukin (IL-) 3. The phenotype of T cells and cytokine expression were quantified by *in vivo* cytokine capture assay, intracellular staining and quantitative RT-PCR. The weight of the mice, histological scores, colon mRNA levels and flowcytometric analysis of infiltrating cells were used to measure disease activity. *In vitro* basophils were isolated by FACS-sorting (using the markers FcεR1 and CD49b) and incubated with activated T cells.

Results: We show that adoptively transferred T cells rapidly proliferate, produce large amounts of IL-3 and expand the number of basophils. These basophils modify the phenotype of T cells during early expansion, counter-regulate disease inducing Th-1 responses and control the development of colitis. Depletion of basophils results in long lasting upregulation of proinflammatory Th-1 responses and exacerbation of colitis, while expansion of basophils with IL-3 almost completely blocks Th-1 cytokines and improves colitis. *In vitro*, basophil derived IL-4 or IL-6 is able to inhibit IFN gamma and IL-2 production in T helper cells, while only the combined release of both cytokines also suppresses TNF production.

Conclusions: These data show a beneficial role of basophils in a T cell driven model of autoimmunity and identify basophils as potential target in inflammatory bowel diseases.

P0501

$CD4^+$ T cell homeostasis in a monogenic autoimmune disease APECED

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Purpose/Objective: Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a recessive autoimmune syndrome caused by loss-of-function mutations in the Autoimmune Regulator (AIRE) gene. The clinical picture includes autoimmunity targeting especially endocrine organs, chronic mucocutaneous candidiasis and various ectodermal defects. AIRE is a transcriptional factor participating in negative selection of thymocytes and maintenance of peripheral deletional tolerance, but the details of the pathogenesis remain unknown. In APECED the peripheral $CD8^+$ T cell homeostasis is imbalanced and associated with elevated levels of a homeostatic cytokine interleukin-7 (IL-7). In this study we display properties of $CD4^+$ T cell population in APECED.

Materials and methods: Peripheral blood mononuclear cells from APECED patients and healthy controls were stimulated with anti-CD3 antibody, permeabilized and stained with fluorescent dyes for flow cytometric analysis. IL-7 plasma levels were determined by ELISA.

Results: In patients the number of $CD4^+$ $CD45RA^-CCR7^-$ cells, considered effector cells, is increased while the number of $CD4^+$ $CD45RA^+CCR7^+$ cells, considered naïve cells, is diminished. Also the number of recent thymic emigrants (RTE), defined as $CD45RA^+CD31^+$ cells and thought to reflect the thymic conditions, is diminished. Patients' $CD4^+$ population exhibits an increased expression of interferon gamma and interleukin-4, markers of type 1 and type 2 helper T cells respectively. The expression of interleukin-17 was marginal both in patients and controls. No cells expressing two intracellular cytokines at the same time were detected. The alterations in cytokine levels are particularly marked in the $CD4^+$ RTE population. The expression of IL-7 receptor in $CD4^+$ cells is decreased and inversely proportional to the elevated plasma IL-7 concentration in patients. This is associated with increased proliferation rate and decreased CCR7 expression in $CD4^+$ $CD45RO^-$ population.

Conclusions: In APECED the $CD4^+$ cells are hyperreactive. Their differentiation begins prematurely as RTE cells show characters of type 1 or type 2 helper T cells. The perturbations in $CD4^+$ population are related to IL-7 dysregulation. This is of particular interest because IL-7 axis has been linked to other multifactorial autoimmune diseases such as multiple sclerosis.

P0502

CD40L expression identifies human and mouse $CD8^+$ helper T cells

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Purpose/Objective: CD40L expression on activated $CD4^+$ T-*helper*-cells (T_h) is one of the most potent signals for T-cell depended activation of APC and is recognized generally as a hallmark for T_h cells. Recently, we detected that CD40L is expressed also by a major memory subset of $CD8^+$ T cells, which concomitantly are not cytotoxic, shown by lack of Granzyme B, Perforin and degranulation (CD107a).

Materials and methods: human & mouse cells.

Results: In various immune responses against pathogens as *Listeria monocytogenes*, LCMV or *Plasmodium berghei* as well as SV40 T antigen expressing tumor cells we detected up to 50% CD40L expressing $CD8^+$ T cells among the antigen-specific $CD8^+$ T-cell populations. In peripheral blood of healthy human donors on average 25% of memory $CD8^+$ T cells express CD40L. These $CD40L^+$ $CD8^+$ T cells display plasticity with respect to their cytokine profile similar to $CD4^+$ T_h cells and accordingly express cytokines as IL-2, IL-4, IL-17 or IFNγ. Furthermore, *in vitro* assays revealed that $CD40L^+$ $CD8^+$ T cells resemble also functional properties of T_h cells and therefore are able to activate properly B cells or DC, shown e.g. by IgG or IL-12 secretion, respectively.

To analyze the impact of CD40L expression on $CD8^+$ T cells *in vivo* we challenged $Rag1^{-/-}$ mice with tumor cells and injected wt or $CD40L^{-/-}$ $CD8^+$ T cells. Application of wt $CD8^+$ T cells prevented the establishment of a solid tumor, whereas injection of $CD40L^{-/-}$ $CD8^+$ T cells alone resulted in a non-controlled tumor progression similar to non-treated tumors demonstrating that $CD8^+$ T-cell mediated immunity can be heavily impaired in the absence of $CD8^+$ T-cell inherent CD40L expression.

Conclusions: Our results disclose an essential functional relevance of CD40L expressed by $CD8^+$ T cells. Especially, in situations of reduced

$CD4^+$ T-cell help, MHC-II antigen presentation or in tumor-driven tolerogenic conditions with limited danger signals $CD40L^+$ $CD8^+$ T cells may exert essential helper responsibilities for immunity and thus are potent T cells to execute or support effective anti-tumor or anti-pathogen immune therapies.

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P0503

Characterisation of IL-10-producing $CD4^+$ T cells induced by IL-27: implications in autoimmune inflammation?

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Purpose/Objective: Interleukin 27 (IL-27) is a heterodimeric cytokine that was originally believed to be pro-inflammatory by polarising naïve $CD4^+$ T cells to a Th1 cell phenotype. IL-27 has more recently been shown to exhibit a range of potent anti-inflammatory effects on T cells, including the up-regulation of Interleukin 10 (IL-10) production in Interferon-γ^+ (IFN-γ^+) T cells. However there is ambiguity regarding the phenotype and function of IL-10^+ IFN-γ^+ T cells and the role these cells may play in autoimmune diseases such as Experimental Autoimmune Encephalomyelitis (EAE), an animal model of Multiple Sclerosis (MS).
Materials and methods: $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 antibodies *in vitro* for up to 72hrs in non-polarising (NP), Th1, iTreg and Tr1 cell polarising conditions in the presence or absence ($^{+/-}$) of IL-27 and analysed by flow cytometry and ELISA. For *in vivo* investigations, SJL mice were immunised with Proteolipid Protein $_{139-151}$ ($PLP_{139-151}$) in Complete Freunds Adjuvant (CFA) to induce Relapsing Remitting EAE (RR-EAE), and were examined for the presence of IL-10^+ IFN-γ^+ T cells at multiple phases of disease in the periphery and CNS.
Results: IL-10 producing $CD4^+$ T cells driven by IL-27 exhibited high expression of T-bet while lacking Foxp3 and IL-21 (associated with iTreg and Tr1 cells respectively). By ELISA, IL-27 up-regulated IFN-γ and IL-10 secretion, while inhibiting IL-5, another Tr1-associated cytokine. This phenotype was enhanced under Th1 polarising stimuli. *In vivo*, IL-10^+ IFN-γ^+ $CD4^+$ T cells were detectible in low numbers in spleen, lymph node and in spinal cord at multiple disease phases of EAE with variation according to disease phase.
Conclusions: In conclusion these studies show that IL-10^+ $CD4^+$ T cells induced by IL-27 *in vitro* express markers of Th1 cells. *In vivo*, IL-10-producing Th1 cells were present in the periphery and CNS of mice with RR-EAE. Taken together our findings suggest that IL-27 drives the development of IL-10-producing Th1 cells which may modulate autoimmune inflammation.

P0504

Chemoimmunotherapy of MC38 mouse colon carcinoma: cyclophosphamide and dendritic cell vaccine treatment induces the differentiation of $CD4^+$ lymphocytes

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Purpose/Objective: The study aimed to determine the $CD4^+$ cell differentiation induced by the administration of the chemotherapeutic agent and dendritic cell (DC)-based vaccines in MC38 colon carcinoma-bearing C57BL/6 mice.
Materials and methods: C57BL/6 mice with advanced MC38 tumor were injected with cyclophosphamide (CY). After three days, mice were injected with vaccines consisted of bone marrow-derived dendritic cells pulsed with tumor lysate (BM-DC/TAg) and/or dendritic cells of JAWS II line genetically modified for IL-2 production (JAWS II/IL-2) or with control gene JAWS II/Neo. Cell vaccines were administrated in three consecutive weeks. Tumor growth delay over control was estimated. On the 7th day after the last injection, spleens were harvested and splenocytes were stimulated with Concanavalin A (ConA). The percentage of $CD4^+$ cells expressing transcription factors characteristic for lymphocytes Th1, Th2, Th17 and Treg in stimulated splenocytes was analyzed. The production of interferon γ (IFN-γ), interleukin (IL-)4 and IL-17A by cells stimulated with ConA was evaluated.
Results: Administration of cyclophosphamide followed by dendritic cell vaccines caused tumor growth delay compared to CY alone. Chemoimmunotherapy increased the percentage of Th1, Th2 and Th17 and decreased the percentage of Treg cells among $CD4^+$ splenocytes stimulated with ConA, especially when vaccines contained BM-DC/TAg^+ genetically modified JAWS II cells. Additionally, this combined therapy resulted in increase in IFN-γ, IL-4 and IL-17A production by stimulated spleen cells.
Conclusions: Taken together, these findings suggest that administration of CY followed by DC-based vaccines induces the differentiation of Th1, Th2 and Th17 splenocytes and cause decrease in percentage of Treg cells in $CD4^+$ spleen cells. Observed changes in the number of $CD4^+$ T cell of each subsets during chemoimmunotherapy may be valuable prognostic factor and a basis for future improvements of anticancer therapy

The financial support of the study is granted by the National Science Center of Poland (decision number DEC-2011/01/N/NZ4/01725).

P0505

Comparison of effector and regulatory cytokine expression of T cells in the target organ of experimental monophasic and relapsing autoimmune disease

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Purpose/Objective: Human autoimmune diseases usually present with a chronic or relapsing course, while most animal models are monophasic. We could establish a rat model of relapsing autoimmune uveitis (EAU), an intraocular inflammatory disease, which enables us to investigate the underlying immune reactions. We compared the intraocular immune cell populations isolated during the course of relapsing with that of monophasic EAU.
Materials and methods: Rats were immunized with retinal soluble antigen (S-Ag) peptide PDSAg (inducing monophasic EAU) or interphotoreceptor retinoid-binding protein (IRBP) peptide R14 (inducing relapsing EAU) in CFA. Intraocular cells were collected at various time points during ocular inflammation and stained *ex vivo* for TCR-$\alpha\beta$ and intracellular IFN-γ, IL-17, IL-10 and Foxp3 expression.
Results: During the course of monophasic uveitis intraocular T cells coexpressing IFN-γ with IL-17, as well as IFN-γ or IL-17 with IL-10, respectively, increased. In contrast, in relapsing EAU the number of IL-17^+ , IFN-γ^+IL-17^+ and IL-17^+ IL-10^+ cells decreased at resolution, while IFN-γ^+ cell numbers remained elevated also during relapses. In general, only cell populations that express only one of the tested cytokines slightly increased or remained stable during recurrences. $Foxp3^+$ cells increased in both, monophasic and relapsing EAU.
Conclusions: The change of the intraocular T cell populations during EAU and the large numbers of T cells producing multiple cytokines point to a strong population dynamics in the eye. We observed differences between monophasic and relapsing disease with respect to IFN-γ^+ and IL-17^+ populations. The strong increase of IL-10^+ T cells in

the eyes during monophasic EAU suggests a regulatory role of these cells with a potential to prevent relapses, rather than Foxp3 expressing cells, which increased in both monophasic and relapsing EAU.

P0506
Der P 1 induces FOXP3$^+$ GATA3$^+$ T cells in allergic individuals

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Purpose/Objective: Functionally distinct T helper cell subsets such as regulatory T cells (Treg) and T helper 2 (Th2) cells play an important role in allergy. Originally FOXP3 and GATA3 were shown to be the master transcription factors for Treg and Th2 cells, respectively. However accumulating evidence, mainly from murine models, indicates that these transcription factors are not restricted to a single T cell lineage and GATA3 expression has been implicated in controlling Treg physiology under inflammatory conditions. So far data on the differential function of these transcription factors in humans are lacking and it remains to be investigated whether these have additional functions in human disease. We studied the *in vitro* induction of FOXP3 and GATA3 upon antigen-specific activation.
Materials and methods: We stimulated peripheral blood mononuclear cells (PBMC) of allergic children and non-sensitized healthy controls with allergens like Der P1 and Bet V1. After various days of culture cells were analyzed for transcription factors GATA3 and FOXP3, phenotypic markers and different cytokines to evaluate differences in Th2 and Treg responses.
Results: In allergic individuals, besides a pronounced Th2 response with high GATA3, IL4 and IL13 expression, a select population of cells was found to co-express both GATA3 and FOXP3. These double positive cells were highly proliferative upon allergen stimulation and produced Th2 cytokines, but at lower levels than the GATA3 single positive cells. In addition, they were able to potently suppress T cell proliferation and the production of IFNgamma and TNFalpha *in vitro*.
Conclusions: In conclusion, we identified a unique allergen-specific CD4$^+$ T cell population that co-expresses GATA3 and Foxp3 and displays functional features of both a Th2 and Treg phenotype. These data further indicate that GATA3 and FoxP3 cannot be used to uniquely define human T helper cell subsets.

P0507
Distinct differentiation requirements and functional properties of microbe-specific human Th17 cells

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Purpose/Objective: Th17 cells have emerged as a new T helper cell lineage involved in the clearance of extracellular bacteria and fungi. A dys-regulated Th17 response, however, can induce severe tissue destruction and autoimmunity. Therefore, mechanisms must be in place to shield the host from immune-mediated damage. In this study our aim was to analyze mechanisms by which human Th17 cell responses could be restrained in settings of infections.
Materials and methods: We developed an *in vitro* assay for the generation of human antigen specific T helper cells using whole microbial antigens.
Results: We demonstrate that human Th17 cells transiently produce the anti-inflammatory cytokine IL-10 upon stimulation. Interestingly, IL-10 expression was accompanied by reciprocal down-regulation of IL-17, leading to a functional regulatory Th17 cell phenotype after the peak of the effector response. The ability of Th17 cells to express IL-10 was, however, restricted to certain antigen specificities. *Ex vivo* isolated *C. albicans* specific Th17 cells could not produce IL-10 in comparison to *S. aureus* specific Th17 cells. This was due to differential priming requirements of these Th17 cell sub-populations. IL-1beta instructed naïve T cells to develop into a pro-inflammatory non-IL10 expressing Th17 cell subset. Th17 cell priming with *S. aureus*, however, was not IL-1beta dependent, leading instead to the generation of IL-10 producing Th17 cells with self-regulatory activities.
Conclusions: Thus, using a novel approach that combines the *in vitro* priming of naïve T cells by whole microbes with the *ex vivo* analysis of memory T cells we were able to unmask the existence of two types of Th17 cells that differ in priming requirements, TCR repertoire and function. This approach revealed that IL-1beta is a molecular switch for determining a functional memory for IL-10 expression. This has important consequences for the physiological termination of pro-inflammatory immune responses and the limitation of bystander damage in certain pathogen microenvironments. Targeting IL-1beta early in the differentiation process of Th17 cells (as we demonstrate with IL1ra therapy in CAPS patients) might therefore represent a promising therapeutic strategy to confer anti-inflammatory properties to cellular mediators of autoimmune diseases.

P0508
Distinctive features of classic and non-classic (Th17-derived) human Th1 cells

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Purpose/Objective: T helper 17 (Th17) lymphocytes represent a third arm of the CD4$^+$ T cell effector responses in addition to Th1 and Th2 cells. Th17 cells have been found to exhibit high plasticity because they rapidly shift into the Th1 phenotype in the inflammatory sites. In human beings, Th1 cells derived from Th17 cells express CD161, whereas classic Th1 cells do not, and they have been named as non-classic Th1 cells.
Materials and methods: In this study, we examined similarities and differences between classic and non-classic human Th1 cells by assessing a panel of T-cell clones, as well as CD161$^+$ or CD161$^-$CD4$^+$ T cells derived *ex-vivo* from the circulation of healthy subjects or the synovial fluid of patients with juvenile idiopathic arthritis.
Results: The results showed that non-classic Th1 cells could be identified because of CD161 expression, as well as the consistent expression of retinoic acid orphan receptor C, IL-17 receptor E, CCR6 and IL-4-induced gene 1, which were all virtually absent in classic Th1 cells.
Conclusions: The possibility to distinguish these two cell subsets by using such a panel of markers may allow the opportunity to better establish the respective pathogenic role of classic and non-classic (Th17-derived) Th1 cells in different chronic inflammatory disorders.

P0509

GM-CSF production by $CD4^+$ T cells from MS patients

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Purpose/Objective: Th17 cells are believed to be the main pathogenic T cells in multiple sclerosis (MS). Recent studies in experimental models of MS suggest that GM-CSF producing T cells might be even more pathogenic. Data on GM-CSF in MS patients are scarce. We hypothesize that GM-CSF expression is elevated within the $CD4^+$ T cell compartment of MS patients.
Materials and methods: PBMC and $CD4^+$ T cells were isolated from MS patients ($n = 31$ and $n = 33$, respectively) and healthy controls (HC; $n = 21$ and $n = 20$, respectively). GM-CSF mRNA expression was assessed by real-time quantitative PCR and is expressed relative to GAPDH expression levels. Intracellular flow cytometry was performed on PBMC from HC and MS patients (untreated, glatiramer acetate (GA) or interferon beta (IFN-β) treated patients) to determine the fraction of $GM\text{-}CSF^+$ cells within the $CD4^+$ T cell compartment. Differences between cohorts were assessed with the Mann-Whitney U-test and Bonferroni correction was used to compensate for multiple testing. A P-value <0.025 was considered significant.
Results: GM-CSF mRNA expression levels are higher in PBMC as compared to $CD4^+$ T cells ($n = 52$; $24.2-10^{-3}$ [9.5×10^{-3}—45.5×10^{-3}] and $n = 53$; 9.1×10^{-4} [5.6×10^{-4}—13.3×10^{-4}], respectively; $P < 0.0001$). GM-CSF mRNA levels do not differ between MS patients and HC in PBMC (24.0×10^{-3} [9.2×10^{-3}—43.9×10^{-3}] and 24.4×10^{-3} [9.9×10^{-3}—46.9×10^{-3}], respectively) or in $CD4^+$ T cells (7.6×10^{-4} [5.6×10^{-4}—1.2×10^{-4}] and 10.9×10^{-4} [5.4×10^{-4}—1.6×10^{-4}], respectively). Preliminary data show that, compared to HC, the $GM\text{-}CSF^+CD4^+$ T cell percentage is elevated in MS patients without treatment. Compared to HC, the fraction of these cells was comparable in MS patients on IFN-β treatment and lower in MS patients treated with GA.
Conclusions: GM-CSF mRNA expression levels are comparable between MS patients and HC. Preliminary data suggest that the $GM\text{-}CSF^+CD4^+$ T cell percentage in the circulation is elevated in MS patients compared to HC. Medication, especially GA, seems to be able to reduce $GM\text{-}CSF^+CD4^+$ T cell numbers.

P0510

Heterogeneous concentrations of interleukin-27 and the environment specific effects of interleukins-17 and -27 in humans

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Purpose/Objective: Data suggest both pro-and anti-inflammatory functions for IL-27. We therefore investigated the role of IL-27 in humans, with particular emphasis on its heterogeneity and relationships with IL-17.
Materials and methods: IL-27 levels by ELISA in (i) plasma of 879 healthy humans from 163 families (ii) after induction by IL-17 from PBMC.
Results: Concentrations were heterogeneous, spanning two orders of magnitude.Heritability (19%) was relatively low, indicating most variation was environmentally determined.As ~10% individuals had high levels, one of the subunits of IL-27 is Epstein-Barr virus -induced gene 3 (EBI3) and ~5—10% of individuals are uninfected with the virus, we tested whether prior infection with EBV, or CMV, was associated with IL-27 values but little correlation was seen.IL-27 has been reported to inhibit IL-17 production, so we also measured IL-17 values in a subset of individuals and, surprisingly, demonstrated a strong positive correlation, but low correlations with other pro-inflammatory cytokines. We also verified that IL-27 inhibited IL-17 production *in vitro*, but were unable to confirm its stimulation of IL-10 secretion.
Conclusions: IL-27 levels are very heterogeneous in human populations, but correlate with IL-17 levels, which suggests how the two cytokines might interact*in vivo*.

P0511

Human T helper 17 responses: the effect of T-cell density

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Purpose/Objective: Both the cytokine milieu and quality of T-cell activation are capable of determining effector $CD4^+$ T-cell development. We recently found that low strength T-cell activation promotes T helper 17 (Th17) cell responses via a Ca^{2+} /NFATc1 dependent mechanism. Here we have extended these investigations by assessing the effect of T-cell density, on Th17 cell responses. T-cell density is a parameter often overlooked within *in vitro* investigations, but is capable of modulating many parameters including the expression of certain Th17 cell related transcription factors.
Materials and methods: Memory $CD4^+$ T-cells were activated with anti-CD3/anti-CD28 beads at a high strength stimulus of 1:1 cell:bead ratio, in the presence of pro-Th17 cell cytokines IL-1β, TGF-β, and IL-23. Cell density was decreased from 1×10^6 to 0.0625×10^6 cells/ml, maintaining a 1:1 cell:bead ratio. After 6 days the proportion and number of IL-17- and IFN-g-producing cells was determined by intracellular cytokine staining and flow cytometry.

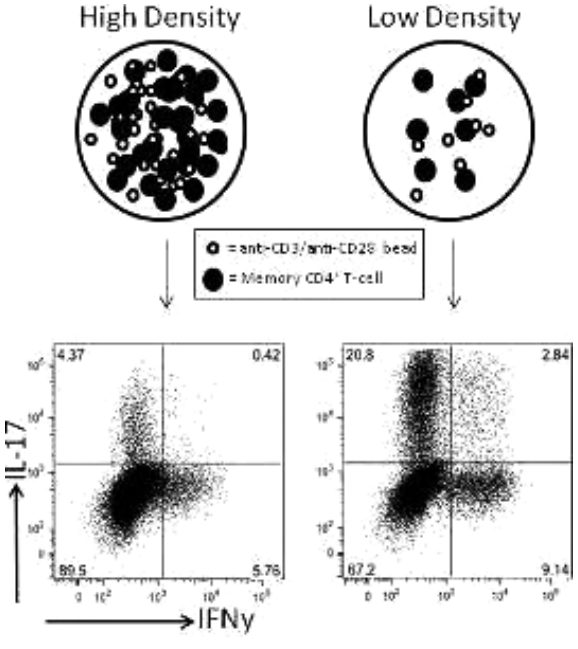

Figure 1. Low T-cell density promotes Th17 responses.

Results: Our results demonstrate that low cell density significantly increased both the proportion and absolute numbers of IL-17$^+$ cells. Titration of pro-Th17 cell cytokines revealed that the effect was not due to excess cytokine availability. Furthermore, Th1 cell responses, in the presence of IL-12, were unaffected by cell density, suggesting an IL-17-selective effect of cell density. STAT3 and Aryl hydrocarbon Receptor (AhR) are two transcription factors involved in promoting Th17 cell responses. Previous studies have indicated that STAT3 and AhR activation and expression may, in part, be dictated by cell density. We found an increased level of STAT3 phosphorylation and AhR expression in low density T-cells, compared to high density T-cells, possibly explaining how activation of T-cells at low density favours Th17 cell responses.
Conclusions: These findings provide new insights into the complex range of factors capable of affecting Th17 cell responses, and have important implications for *in vitro* differentiation models.

P0512
IL-17 signaling is related to disease severity in chemically induced type 1 diabetes

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Purpose/Objective: IL-17-secreting CD4$^+$ T cells play an important pathogenic role in several models of autoimmune diseases. However, their involvement in diabetes development is still not well understood, and controversial data are present in the literature. The aim of this study was to examine the role of IL-17 during the initial phase of diabetes development.
Materials and methods: The diabetes was induced in C57BL/6 (WT) and IL-17 receptor deficient (*Il17r* KO) mice after five consecutive injections of streptozotocin (STZ). Pancreas and pancreatic lymph nodes were harvested six days after the last dose of STZ. Pancreatic lymph nodes were analyzed by flow cytometry and the histopathological alterations in the pancreatic islets were evaluated by H&E and immunohistochemistry staining.
Results: Six days after STZ administration, we observed that *Il17r* KO mice showed decreased levels of blood glucose when compared to diabetic WT mice (241 ± 25.65 mg/dl versus 337.2 ± 24.06 mg/dl, respectively). To assess whether this resistance was accompanied by a reduced islet inflammation, pancreatic sections from *Il17r* KO and WT were analyzed. Histological results showed that diabetic WT mice exhibited typical islet inflammation, whereas *Il17r* KO islets had none or few inflammatory cells. In addition, we observed a preservation of beta cells mass in *Il17r* KO mice, demonstrated by insulin staining, when compared to WT mice. To explore the possibility that alterations in regulatory T cells or IFN-γ-producing T cells might play a role in the resistance of the *Il17r* KO mice to the development of diabetes we assessed the frequency of these populations in the spleen and pancreatic lymph nodes. We observed that *Il17r* KO mice exhibited increased frequencies of regulatory T cells (CD4$^+$ CD25$^+$ Foxp3$^+$) in PLNs when compared to WT mice. We did not observe differences in CD3$^+$ CD4$^+$ IFN-γ^+ or CD3$^+$ CD8$^+$ IFN-γ^+ populations in spleen and PLNs.
Conclusions: Thus, differences in disease severity cannot be explained by alterations in IFN- γ -producing T cells. However, the IL-17 signaling appears to counter regulate the regulatory T cell induction in PLNs and influence the diabetes development. Finally, the IL-17 signaling is involved in the initiation of the early events of beta cell destruction in STZ-induced diabetes model.

P0513
IL-21 limits human T helper effector cell differentiation by antagonizing IL-2 signaling

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Purpose/Objective: Induction and regulation of follicular T helper (Tfh) cells are still a matter of debate. IL-21 has been shown to maintain the Tfh subset, whereas IL-2 signaling inhibits Tfh differentiation. However, regulation of Tfh after migration into the germinal center is not completely understood, since regulatory T cells are unable to migrate into the germinal center. With this study we further investigate the role of IL-21 and IL-2 in the regulation of effector functions of human T helper cells.
Materials and methods: We used *Salmonella*-infected B cells to induce optimal Tfh differentiation of naïve and memory T cells, and studied T helper cell activation and differentiation by intracellular cytokine staining and mRNA transcription levels.
Results: Using *Salmonella*-infected human B cells, we show that the Tfh cytokine IL-21 regulates the contraction phase of activated CD4$^+$ T cells. Although IL-21 enhances T cell proliferation at the beginning of activation, proliferation and differentiation is arrested at later stages. We found that IL-21 counteracts effector cell formation through inhibition of endogenous IL-2 synthesis. In line with this, addition of IL-2 almost completely restores proliferation and Th differentiation in presence of IL-21.
Conclusions: Thus, Tfh can downmodulate the immunresponse via secretion of IL-21, preventing hyper inflammatory responses in the germinal center.
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P0514
IL-4 negatively regulates IL-5 memory expression in T helper type 2 cells

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Purpose/Objective: T helper type 2 (Th2) cells are characterized by their expression of interleukin-4 (IL-4), IL-5, and IL-13. It has been well established that for the expression of these cytokines the transcription factor GATA-3 is required. While the memory for IL-4 reexpression in Th2 cells has been intensively studied, regulation of IL-5 and IL-13 reexpression is less clear. Thus, we have analyzed the stability and plasticity of IL-5 memory expression in Th2 cells.
Materials and methods: In order to analyze the IL-5 expression on single cell level, we have isolated IL-5 expressing cells with the IL-5 cytokine secretion assay from *in vitro* generated Th2 cells. We have analyzed the fidelity of IL-5 reexpression in the presence or absence of the Th2 instructive signal IL-4 and have also analyzed the IL-4 dependence of IL-33 induced IL-5 expression.
Results: Our results show that although IL-5 expression is induced via the IL-4/STAT6/GATA-3 axis, blockade of IL-4 with anti-IL-4

antibodies during subsequent reactivation greatly enhanced the frequency of Th2 cells expressing IL-5. IL-5 memory expression was directly inhibited as IL-4 inhibited the reexpression of IL-5 in Th2 cells previously sorted based on IL-5 expression. IL-4 also inhibited IL-5 expression in Th cells which ectopically overexpressed GATA-3. The presence or absence of IL-4 did not influence the reexpression of IL-4 or the expression of GATA-3 in Th2 cells. In addition we could also demonstrate that IL-5 expression induced by stimulation of Th cells in the presence of IL-33 was also inhibited by IL-4.
Conclusions: We could show that the memory expression of IL-5 in Th cells is inhibited by the presence of IL-4. Thus, we have identified a negative regulatory feedback mechanism which limits the expression of IL-5 in memory Th2 cells through autocrine IL-4 signaling. This could present a mechanism which limits IL-5 mediated immunopathology in Th2 immune responses.

P0515

Inhibition of TNFRII signalling by anti-TNFa polarizes naïve $CD4^+$ T cell towards IL-10^+ cells with strong regulatory phenotype and function

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Purpose/Objective: TNFα is a potent pro-inflammatory cytokine playing a pivotal role in several autoimmune diseases. Neutralizing TNFα inhibits T cell proliferation and IFNγ production, and enhances suppressive capacity of regulatory T cells (Treg). Little is known about the effect of TNFα blocking agents on naïve T cell differentiation.
Materials and methods: In order to investigate the role of TNFα in T cell polarization, we blocked TNFα with adalimumab, a human monoclonal antibody against TNFα. We have previously shown that IL-10-generated tolerogenic (t) DC are excellent inducers of regulatory T cells (Treg) that strongly suppress T cell reactivity. As IL-10 tDC produce high amounts of the pro-inflammatory cytokine TNFα, we used these DC to investigate effects of presence or absence of anti-TNFα on naïve T cells priming. Primed T cells are analyzed by intracellular cytokine staining, micro array analysis and tested for functional suppression in a suppression assay.
Results: Here, we report that neutralizing TNFα during priming of naïve $CD4^+$ T cells favors development of IL-10^+ Th cells at the expense of IFNγ expressing effector Th cells. TNF inhibits IL-10 via TNFRII, which becomes expressed a few days after naïve T cell activation. Neutralization of TNFα did not affect initial $CD4^+$ T cell activation, but negatively affected later stages of T cell priming by counteracting full T cell differentiation and increasing cell death. Whole genome gene expression analysis revealed a regulatory gene profile of anti-TNFα-treated T cells. Indeed, neutralizing TNFα during naïve T cell priming enhanced the suppressive function of the anti-TNFα-treated T cells in a functional suppression assay.
Conclusions: Taken together, inhibition of TNFα*TNFRII interaction affects late stage effector T cell development and shifts the balance of Th differentiation towards immune regulation, which might be beneficial in TNFα blocking therapies.

P0516

Interferon gamma protects mice from CNS autoimmunity also in the absence of IL-17A and IL-17F

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Purpose/Objective: The role of interferon (IFN)-gamma-producing T helper (Th) 1 and interleukin (IL)-17-expressing Th17 lymphocytes in multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE) is still subject to intense investigations. Here we sought to decipher the specific involvement of these Th cell subsets during manifestation and the clinical phase of EAE.
Materials and methods: A major hallmark of encephalitogenic Th17 cells is the expression of IL-17A and IL-17F, which together form the heterodimeric effector cytokine. Therefore, we used a double-knockout mouse lacking both isoforms of IL-17 (IL-17AFKO). Additionally, this mouse was crossed to the routinely used IFN-gamma deficient mouse, resulting in triple-knockout IL-17AFKO/IFNgKO animals. Mice were subjected to EAE induction with monitoring of the disease course and subsequent flow cytometric analysis of CNS infiltrates.
Results: As demonstrated before, following active MOG peptide immunization, IFNgKO mice developed an exacerbated EAE disease course compared to wild type C57Bl/6 controls. In contrast, IL-17AFKO animals failed to induce prominent disease which was accompanied by a dramatic reduction of CNS-infiltrating immune cells. This phenotype could be partially reversed in triple-knockout animals as these mice showed a delayed onset of EAE with severity of disease reaching the level of wild type controls. Essentially, these results were confirmed in a model of passive EAE where *in vitro*-restimulated mutant T cells were adoptively transferred into wild type recipients. Transfer of IFNgKO as well as triple-knockout cells resulted in disease of comparable levels whereas IL-17AFKO cells failed to induce any signs of EAE.
Conclusions: These results argue for a non-redundant role of IL-17 (AF) for the induction of experimental neurodegenerative disease. Moreover, the beneficial role of IFN-gamma in EAE seems to be independent of the action of IL-17. Finally, our results clearly point towards T cells as carriers of these effects.

P0518

Level of CD44 activation marker expression distinguishes allo-reactive TH17 from TH1 cells

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Purpose/Objective: CD44 is the most prominent activation marker which distinguishes memory and effector from naïve T cells. It is rapidly up-regulated upon T cell stimulation. CD44 is a surface protein and functions as adhesion protein, which mediates rolling over endothelial cells and thereby infiltration into inflamed tissue (for review see Baaten et al., 2012). Interestingly, Il-17 producing T cells dominate over IFNγ$^+$ TH1 cells in peripheral tissues such as lung, liver and gut (Park et al., 2006; Weaver et al., 2009). Here we compared the required TCR signaling strength and activation marker expression of allo-reactive IL-17 or IFNγ producing T cells.
Materials and methods: CD4 MACS sorted cells from lymph nodes and spleen of C57Bl/6 mice were co-cultured with allogeneic bone marrow derived DCs (BMDCs) from BALB/c mice for four days and analyzed by flow cytometry.
Results: Interestingly, covalently labeling of $CD4^+$ T cells with proliferation dye such as CFSE prior to co-culture with allogeneic BMDCs reduced the frequency of CD44 high and IL-$17A^+$ T cells. Similarly, pre-treatment of $CD4^+$ T cells with cytochalasin, which inhibits TCR synapse formation and thus signaling, leads to a decrease in IL-17^+ T cells while IFNγ producing T cells remain nearly unaffected. In order to further investigate whether generation of IL-17^+ T cells needs a strong T cell activation we pre-incubated the BMDCs with increasing amounts of LPS. Indeed, the percentage of IL-17^+ T cells increases with the level of LPS-induced MHCII/CD86 expression on DCs. Furthermore, while $IL17^+$ cells show a very high CD44 expression, IFNγ$^+$ cells are characterized by intermediate CD44 expression. Moreover, co-culture of T cells under TH17 polarizing

conditions increases the percentage of CD44 high T cells, while TH1 polarization results in a reduction of high CD44 expressing T cells. Preliminary results revealed that CD44 low expressing cells accumulate mainly in blood and spleen, but not in peripheral tissues.
Conclusions: Taken together our data demonstrate that a high TCR signaling strength results in an increased formation of IL-17$^+$ T cells. Moreover, allo-reactive IL-17$^+$ cells express high levels of CD44 while IFNγ^+ cells show reduced CD44 expression. Strong CD44 expression on T cells increases the ability to migrate to tissues.

P0519
NFATc2 and c-Jun have unique and non-redundant functions in regulation of chromatin conformation at the TNF promoter in T helper lymphocyte subsets

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Purpose/Objective: Tumor necrosis factor (TNF) is one of the key primary response genes in the immune system activated by variety of stimuli. The purpose of this study was to analyze chromatin conformation at the TNF promoter region in different subsets of T helper (Th) lymphocytes, assuming that it may be different from one found in macrophages.
Materials and methods: For the analysis of chromatin conformation at the TNF promoter in mouse T cells we probed DNA accessibility in chromatin with restriction nucleases and micrococcal nuclease. Quantitative RT-PCR was used for the evaluation of TNF mRNA. Western blotting was applied for the analysis of major transcription factors in nuclear fractions of T cells.
Results: We found that the proximal TNF promoter has a closed chromatin conformation in primary T helper cells and acquires an open state only after activation or polarization under Th1 and Th17, but not under Th2 conditions. Transcription factors NFATc2 and c-Jun are required for such chromatin remodeling at the proximal TNF promoter in T cells, but the two appear to have different roles in this process. NFATc2 plays the primary role in the acquisition of open chromatin conformation at the proximal TNF promoter upon activation, while phosphorylated c-Jun is necessary for the maintenance of this open conformation in cells polarized under Th1 and Th17 conditions.
Conclusions: Transcription factors NFATc2 and c-Jun play unique and non-redundant roles in regulation of chromatin conformation at the TNF promoter in T helper cells.

P0520
Novel factors involved in Th17 cell differentiation

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Purpose/Objective: Naive CD4$^+$ T helper (Th) cells can differentiate in the presence of IL6 and TGFb into Th17 cells, which provide immunity against extracellular bacterial and fungal pathogens but are also implicated in pathological responses, such as autoimmune diseases. Aryl hydrocarbon receptor (AhR), also known as a dioxin receptor, is a transcription factor, which is upregulated during the Th17 cell differentiation. Our objective is to identify and define the underlying regulatory mechanisms and signalling networks that determine the steps essential for generating Th17 phenotype.
Materials and methods: We have performed a detailed kinetics study to dissect the gene expression profile of Th17 cells under conventional or modified polarizing conditions and using mouse strains with different affinity of AhR. FACS sorted naïve CD4$^+$ T cells were activated with plate-bound anti-CD3 and anti-CD28. Th17 differentiation was induced with a combination of cytokines TGFb and IL6 and modified with IL1b or IL21 or AhR ligand FICZ. An endpoint assessment of an aliquot for each condition was tested after 72 or 96h for intracellular expression of IL-17A as well as secreted cytokines. The gene expression profiles were analysed using Illumina mouse whole genome beadarrays and mRNA-seq technology.
Results: Preliminary results show that thousands of genes are differentially expressed in the developing Th17 cells compared to naïve or activated T cells. These differentially expressed genes include key transcription factors, such as *Rorc*, *Rora* and *Batf*, Th17 cell hallmark cytokines *Il17a*, *Il17f* and *Il22* as well as characteristic surface markers *Il23R* and *Ccr6*. Some of these molecules are also controlled by AhR as their expression is differentially regulated in AhR$^{-/-}$ CD4$^+$ T cells or by stimulating the WT CD4$^+$ T cells with FICZ.
Conclusions: These results will provide a solid basis for identification of novel factors and signalling pathways crucial for the development of proper Th17 phenotype as well as for further studies aiming at perturbing the Th17 differentiation process.

P0522
Plasmablasts induce human CD4$^+$ T follicular helper cells via secretion of IL-6

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Purpose/Objective: CD4$^+$ T follicular helper (TFH) cells are a distinct lineage specialized in providing B-cell help characterized by the expression of CXCR5, ICOS and the transcription factor Bcl-6. We explored whether B cells reciprocally modulated TFH cells.
Materials and methods: Different subsets of human CD4$^+$ T cells and CD19$^+$ B cells were isolated by FACS sorting from peripheral blood and cultured *in vitro*. TFH cell number and function were assessed by FACS and *in vitro* assays.
Results: *In vitro* depletion of B cells from anti-CD3/28 stimulated PBMCs resulted in a twofold reduction in Bcl-6 expression within the CXCR5$^+$ ICOS$^+$ TFH cell population ($P = 0.0008$). The numbers of TFH cells was significantly increased when responder T cells (CD4$^+$ CD25$^-$CD127$^+$) (Tresp) were stimulated (anti-CD3/IL-4) in the presence of B cells (8 ± 0.5 [with B cells] versus $4 \pm 0.8\%$ [no B cells], $P = 0.0002$). In these co-cultures the percentage of TFH cells correlated with the percentage of plasmablasts (CD19$^+$ CD38^{high} CD27^{high}) ($P = 0.0456$).Moreover, purified plasmablasts were significantly more potent in increasing the number of TFH cells in these cultures ($8 \pm 1\%$) compared to naïve B cells (CD19$^+$ CD38$^{-/low}$CD27$^-$) ($3 \pm 0.7\%$, $P = 0.01$).In addition, plasmablasts were more potent inducers of functionally active TFH cells from naïve CD4$^+$ T cells (CD45RA$^+$CD27$^+$) ($18 \pm 2\%$) than naïve B cells ($10 \pm 1\%$ $P < 0.0001$).*In vitro*, induction of TFH cells from naïve CD4$^+$ T cells was significantly correlated with *in vitro* IL-6 production ($P = 0.001$) and plasmablasts produced more IL-6 compared to naïve B cells (5 ± 1 versus $1 \pm 0.4\%$, $P = 0.05$).Blockade of IL-6 in co-cultures of naïve CD4$^+$ T cells and plasmablasts significantly decreased acquisition of a TFH cell phenotype ($P = 0.0013$). RA patients have significantly more circulating TFH cells compared to healthy individuals ($P = 0.003$) and consistent with our *in vitro* data, RA patients responding to Tocilizumab (IL-6R blockade) therapy showed a marked reduction in circulating TFH cell numbers (pre: 1.2 ± 0.1 versus post: $0.4 \pm 0.06\%$) and IL-21 production, which was associated with reduced plasmablast formation.
Conclusions: In conclusion, our data provide new insights into the reciprocal regulation between human plasmablasts and TFH cells and the pivotal role of IL-6. Tocilizumab reduced TFH cell numbers in

patients with RA highlighting a novel mechanism of action for this therapy.

P0523
Plasticity of CD4 T cells in rheumatoid arthritis

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Purpose/Objective: Th17 cells are a subset of CD4 helper T cells defined by the production of interleukin 17 (IL-17). Th17 cells are physiologically involved in the host defense but are also known to be a major contributor in the pathogenesis of autoimmune diseases. An increased number of Th17 cells has been reported in patients with rheumatoid arthritis (RA). The mechanisms leading to this predominance of Th17 cells in RA are not yet fully understood. Recent evidence suggests that Th cell differentiation is a plastic process implying a change in the phenotype depending on inflammatory conditions. An altered T cell plasticity might therefore contribute to the shift towards the Th17 phenotype observed in RA.
Materials and methods: To verify this hypothesis, naïve CD4 T cells were isolated from the blood of RA patients ($n = 5$) with early, untreated disease and healthy controls (HC) ($n = 5$) and subjected to a first round of differentiation under Th17-inducing conditions followed by a cytokine secretion assay and fluorescence activated cell sorting of Th1 and Th17 cells. Sorted cells were then cultured for a second round of differentiation under Th1- and Th17-inducing conditions. The cytokine secretion profile was assessed by flow cytometry.
Results: Sorting of Th1 or Th17 cells generated pure populations of IFNγ- or IL-17-producing cells. Re-differentiation of Th17 cells under Th1-inducig conditions resulted in a cell population containing up to 80% of IFNγ-producing cells in both RA and HC (83.4 ± 6% versus 76.7 ± 7.7%, respectively). Whithin the IFNγ producing cells the frequencies of IFNγ single producers were 21.2 ± 10.4% in RA and 16 ± 3.3% in HC, while the IFNγ/IL-17 double producers were 62.5 ± 13.8% in RA and 60.3 ± 8.3 in HC. The re-differentiation of Th1 cells under Th17-inducing conditions was comparable between RA patients and HC and resulted in 16.3 ± 18.1% (RA patients) and 17.8 ± 9.8% (HC) IL-17-producing cells, respectively. Interestingly, these newly generated IL-17-producing cells preserved the ability to produce IFNγ.
Conclusions: The data indicate that re-differentiation of Th17 cells into Th1 from RA patients did not differ from that of HC. Whether the deregulated re-differentiation capacity of CD4 T cells in RA might contribute to the increase of Th17 cells in the disease will require further investigation.

P0524
Regulation of IL-31 expression in human T helper cell subsets

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Purpose/Objective: IL-31, a four-helix bundle cytokine of the IL-6 family, was shown to be associated with Th2 mediated pathologies including atopic dermatitis, allergic contact dermatitis and allergic asthma. Since only one study shows that rather activated murine Th2 cells than Th1 cells are the source of IL-31, the present study aims to investigate the regulation of IL-31 expression in human *in vitro* polarized Th cells subsets in more detail.
Materials and methods: Human naïve $CD4^+$ T cells were isolated from buffy coats and cultured under distinct polarizing conditions and IL-31 secretion was analyzed by ELISA. EMSA studies were carried out to identify the transcription factors responsible for *IL31* expression. To assess the role of these transcription factors for *IL31* transcription luciferase reporter gene assays and siRNA-mediated silencing experiments were performed.
Results: Analysis of IL-31 production in Th1, Th2, Th9, Th17 and iTreg cells revealed that IL-31 is predominantly released by IL-4 dependent Th cell subsets, namely Th2 and Th9 cells. Notably, IL-31 secretion from Th9 cells is significantly reduced compared to Th2 cells. Suppression of IL-31 secretion in Th9 cells relies on TGF-β1, the main factor inducing the switch from Th2 to Th9 cells. Whereas TGF-β1 reduces the release IL-31, IL-33 a cytokine described to augment Th2 cytokine secretion enhances the release of IL-31 in Th2 cells. Since IL-4 induces signaling via STAT6 and IL-33 activates the NF-κB pathway, we investigated the role of these molecules for IL-31 expression. EMSA, siRNA silencing experiments and luciferase reporter gene assays clearly demonstrate crucial involvement of both transcription factors in activating *IL31* gene transcription.
Conclusions: We show that IL-31 is secreted by human $CD4^+$ Th cell subsets that develop in the presence of IL-4. With STAT6, NF-κB, TGF-β1 and IL-33 four key factors in IL-31 expression have been identified. Taken together, this study provides new insights in the regulation of IL-31 in human Th2 and Th9 cells.

P0525
T follicular helper cells survive as long-term memory cells

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Purpose/Objective: T follicular helper (TFH) cells represent the subpopulation of $CD4^+$ T cells which provides help for antigen-specific B cells in the germinal centre response. Without TFH cells, neither memory B cells nor long-lived plasma cells can be generated. TFH cells are generated from naïve T cells during an immune response and are imprinted by their master transcription factor Bcl-6. It has been a long-standing question if TFH cells can persist in the body as long-term memory cells or if they die after the germinal centre response has been terminated.
Materials and methods: To answer this question we sorted TCR-transgenic TFH and non-TFH effector cells from an ongoing germinal centre response and transferred them into secondary hosts.
Results: When re-transferred into mice with an ongoing immune reaction, antigen-specific TFH cells preferentially migrated to the B cell follicle, indicating that transferred TFH cells are capable of typical homing and function.

When re-transferred into naïve mice (hence T cells did not receive further signals via the TCR), effector cells rapidly contracted with a small population of both TFH and non-TFH cells surviving as memory cells in peripheral lymphoid organs for at least 4 weeks in the absence of antigen. TFH cells strongly downregulated their signature genes Bcl-6, CXCR5, and PD-1 in the memory phase. However, upon rechallenge with antigen they rapidly upregulated these markers again, and had a strongly enhanced potential to produce IL-4 and IL-21.
Conclusions: TFH cells can survive as long-term memory cells but lose their signature markers. A very high expression of CXCR5 and low expression of CCR7 equips re-activated TFH memory cells with a chemokine receptor profile favouring a quick migration to the B cell follicle. In contrast with non-TFH memory cells, re-activated TFH cells have a cytokine profile to efficiently support B cells in germinal centre reactions once again.

P0526

Temporal and spatial dynamics of IL-10 expression in an *in vivo* tolerance model

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Purpose/Objective: Regulation of the inflammatory response to self antigen is of vital importance to limit immunopathology. Dysregulation of this response can lead to autoimmune diseases such as Multiple Sclerosis (MS).Our present research utilises myelin basic protein (MBP) peptide Ac1-9 specific T-cell receptor (TCR) transgenic Tg4 mice to study EAE (Experimental Autoimmune Encephalomyelitis). We have previously shown that repeated intranasal administration of a high affinity form of the immunodominant epitope of MBP AcA-SQYRPSQR [4Y] induces peripheral tolerance in Tg4 mice.Tolerance is characterised by the induction of anergic, IL-10 secreting, FoxP3-, T-bet$^+$ Th1 derived regulatory T cells (IL-10 Tregs). *In vivo* studies have established that IL-10 is required for tolerance, however, it is still unknown how IL-10 functions temporally and spatially over the course of tolerance induction. In this study, we utilised an IRES-GFP IL-10 transcriptional reporter model, to establish the temporal and spatial dynamics of IL-10 expression *in vivo* in a tolerance model.
Materials and methods: IL-10 GFP reporter mice were created by crossing Tg4 mice with C57BL/6 Tiger mice. Mice were treated intranasally a minimum of ten times with 80 μg [4Y] peptide or PBS, organs isolated and CD4$^+$ cells isolated either 2 hor 3 days following the final treatment.
Results: The kinetics and anatomical distribution of GFP$^+$ CD4$^+$ T cells in tolerised mice will be discussed further below.
Conclusions: IL-10 GFP reporter mice facilitated the detection of IL-10 expression in lymphoid organs at various stages of intranasal tolerance induction. Using this reporter mouse we aim to gain a better understanding of the dynamics of IL-10 production, shown to be crucial for successful peptide immunotherapy in autoimmune disease.

P0527

Th1/Th17 balance is controlled by HIF-1a under Hypoxia

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Purpose/Objective: During an immune response, inflammation can damage tissue microvasculature and reduce cellular oxygen supply. This process is referred as tissue hypoxia. When O_2 availability decreases, the transcription factor HIF-1a, a key metabolic sensor, is stabilized and regulates cellular adaptation to hypoxia. The objective of this project was to test whether HIF-1a regulates T cell fate during inflammation and to define the molecular mechanisms of this control.
Materials and methods: Naive CD4$^+$ T cells were cultured under Th1 and Th17 polarizing conditions and then transferred to 1% (Hypoxic) or 20% (Normoxic) O_2 environment. As strategy, we have used genetically deficient mice, bicistronic vectors allowing the overexpression of selected genes, flow cytometry and real time PCR to investigate the mechanisms involved in T helper adaptation to hypoxia. T cell transfer model of chronic colitis is ongoing to investigate the *in vivo* relevance of our observations.
Results: We found that, unlike Th17 cells, Th1 cells lose their effector function (as assessed by IFN-g production) when cultured under hypoxia. Of note, HIF-1a$^{-/-}$ Th1 cells were insensitive to hypoxia underlining a critical role for HIF-1a in Th1 inhibition by hypoxia. Similarly, the production of IFN-g by IL-10$^{-/-}$ Th1 was not altered in hypoxia, supporting a role for IL-10 probably by sustaining STAT3 phosphorylation. In support of this hypothesis, STAT3 was phosphorylated constitutively in both Th1 and Th17 cells under hypoxia, but not normoxia, and STAT3$^{-/-}$ Th1 cells produced similar amounts of IFN-g in normoxic and hypoxic conditions, suggesting that, in hypoxic Th1 cells, STAT3 may favor HIF-1a accumulation. The mechanism by which HIF-1a decreased Th1 activation could involve the inhibition of STAT4 activation as suggested by our preliminary data.
Conclusions: We found that hypoxic culture conditions result in decreased Th1 activation while sparing Th17. Our results suggest that IL-10 secretion by Th1 cells induces a STAT3 dependent feedback leading to HIF-1a accumulation, which may in turn inhibit the STAT4 signaling pathway.

P0528

The role of Interleukin-6 in the immune responses against Listeria monocytogenes

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Purpose/Objective: The cytokine Interleukin-6 (IL-6) plays a protective role in immune responses against bacterial infections. However, the mechanisms of IL-6-mediated protection are only partially understood. IL-6 can signal via a complex of membrane-bound IL-6Rα (mIL-6Rα) and gp130 (classical signaling). Under some conditions, IL-6Rα is shed from the surface. Soluble IL-6Rα still binds IL-6 and the IL-6/sIL-6Rα complex can interact with gp130 and induce signaling (trans-signaling).
Materials and methods: In this project, we apply the *Listeria monocytogenes* infection model to investigate the role of IL-6 in the immune response against bacterial infection.
Results: Following *L. monocytogenes* inoculation, IL-6Rα was rapidly lost from the surface of all analyzed leukocytes subsets. We also observed decrease of IL-6Rα surface expression in IL-6-deficient mice excluding IL-6 mediated receptor internalization or anti-IL-6Rα mAb blockade. *In vitro*, IL-6Rα could be rapidly shed via a P2X7 dependent mechanism. However, shedding *in vivo* occurred independently of P2X7. Loss of IL-6Rα expression was also not correlated to loss of surface expression of other targets of sheddases such as CD62L or CD27.

Analysis of the acquired immune response against *L. monocytogenes* revealed a failure of IL-6-deficient mice to generate IL-17A producing CD4$^+$ T cells. However, IL-17A production by γδT cells was not dependent on IL-6. Generation of *L. monocytogenes*-specific CD8$^+$ T cells and CD4$^+$ Th1 cells was also not affected by the absence of IL-6.
Conclusions: In summary, our results demonstrate that surface expression of IL-6Rα is highly dynamic during *L. monocytogenes* infection pointing to a role of IL-6 classical signaling and trans-signaling during infection. And although IL-6 is essential for the function of Th17 cells, the generation of *L. monocytogenes*-specific T cells response is largely independent of IL-6.

P0529

The role of the aryl hydrocarbon receptor in T helper 17 polarisation: strain comparisons

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Purpose/Objective: The aryl hydrocarbon receptor (AhR) has been the subject of much research as an environmental toxin receptor. Attention has focused primarily on the response to dioxin, with one of the key discoveries being that differences in physiological and toxic responsiveness can be based on genetic variant forms of AhR with

different affinities for the agent. For instance, discrete strains of mice have different variants of AhR; C57BL/6 being very responsive, BALB/c being mid responsive and DBA/2 strain mice being the least responsive. Interestingly, the human AhR is a low-affinity receptor analogous to the AhR in DBA/2 strain mice. Several recent studies have described that activation of the AhR enhances polarisation of Th17 cells *in vitro*. However, all studies to date have used the high affinity AhR mouse strain C57BL/6.We have, therefore, investigated whether the class of AhR in different strains of mice affects optimal *in vitro* polarisation of Th17 cells.

Materials and methods: Following magnetic bead isolation, $CD4^+$ cells (>95% pure) from C57BL/6, BALB/c and DBA/2 mice were polarised *in vitro* using interleukin (IL)-6, IL-1β and transforming growth factor-β in medium containing a natural AhR agonist (tryptophan). Successful polarisation was determined by production of IL-17 measured by RT PCR and ELISA. To assess the contribution of AhR activation during Th17 polarisation; cells were cultured in the presence or absence of an AhR antagonist (CH-223191; 3 μM).

Results: Th17 polarisation was achieved for all three mouse strains, with the highest levels of IL-17 protein and mRNA expression recorded for C57BL/6 tissue. However, levels achieved for C57BL/6 mice were lower than those reported by other authors. Following inhibition of the AhR using the antagonist, all mouse strains displayed at least a 50% reduction in Th17 polarisation but interestingly, levels of IL-17 mRNA and protein were particularly reduced (>80%) in the low affinity AhR DBA/2 strain mice.

Conclusions: These data suggest that AhR activation is essential for optimal Th17 polarisation in all mouse strains investigated and that considerations of AhR affinity do not apply when considering the influence of a natural agonist on Th17 polarisation.

P0530

The Shc family protein adaptor, Rai, acts as a negative regulator of Th17 cell development

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Purpose/Objective: Rai acts as a negative regulator of antigen receptor signaling in T and B cells. $Rai^{-/-}$ mice develop lupus-like autoimmunity associated to the spontaneous activation of self-reactive lymphocytes. Here we have addressed the potential role of Rai in the development of the proinflammatory Th1 and Th17 subsets.

Materials and methods: Lymph node T cells or naïve T cells were isolated from wild-type and $Rai^{-/-}$ mice and their cytokine profile wasdetermined by ELISPOT and qRT-PCR both as such and after stimulation *in vitro* with immobilized anti-CD3 mAb in the presence or absence of polarizing cytokines. The expression of rai was measured in PBL from SLE patients and healthy donors by qRT-PCR.

Results: We show that $Rai^{-/-}$ mice display a spontaneous Th1/Th17 bias. *In vitro* polarization experiments demonstrate that rai deficiency favours the development and expansion of Th17, but not Th1, cells, indicating that Rai modulates TCR signaling to antagonize the pathways driving naïve $CD4^+$ T cell differentiation to the Th17 lineage. Th1 and Th17 cell infiltrates were found in the kidneys of $Rai^{-/-}$ mice, providing evidence that Rai deficiency contributes to the development of lupus nephritis not only by enhancing lymphocyte activation but also by promoting the development and expansion of proinflammatory effector T cells. Interestingly, T cells from SLE patients were found to have a defect in Rai expression, suggesting a role for Rai in disease pathogenesis.

Conclusions: In conclusion, we have identified Rai as a negative regulator of Th17 cell differentiation and expansion in the mouse and found evidence of an impairment of Rai expression in PBL from SLE patients, where a Th1/Th17 bias has been clearly documented, which suggests that it might subserve a similar function in humans. Given the causal role of Th17 cells in a number of autoimmune disorders, such as multiple sclerosis and rheumatoid arthritis, these data suggest that Rai may play a function in preventing autoimmunity beyond lupus nephritis.

P0531

The signalling strength of Phosphatidylinositol-3-kinase (PI3K) pathway regulates T helper subsets and plays a central suppressive role in autoimmunity

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Purpose/Objective: Recent works have shown the importance of Phospohayidylinositol-3-kinase (PI3K) on T helper cell commitment. Using distinct genetic means we show that the intensity of the PI3K signal generate T helper cells type 1, T helper cells type 17 and induced regulatory T cells subsets.

When thePI3Ks signaling pathwayis constitutively over-expressed on T cells; mice do not develop any signs of induced Experimental Autoimmune Encephalomyelitis (EAE) and of induced colitis.We show then, a new role of the PI3Ks signalthat plays a central role in induction of tolerance on autoimmunity via IL-22 and IFNγ.

Materials and methods: $CD4^+$ CD62LT cells oniTreg, Th1 cells, and Th17 cells: LNs and SPs from WT mice, PD-1 KO, CD4creP110ind mice were harvested and naiveT cells were sorted using Magnetic Beads columns. The cells were cultured from 1 to 7 days under different conditions.

Induction and assessment of EAE: Active EAE was induced by immunization with 50 μg of MOG35*55 peptide emulsified in CFA. Mice also received 200 ng of PTi.p. on the day of immunization and two days later. Clinical assessment of EAE was performed according to the standard criteria: from 0 to 6 based on the level of the sickness.

Results: We demonstrate that different concentration of the CD28 stimulus and/or the lack of the PD-1 receptor inhibits the production of IL-17A under TH17 condition, impairs the generation of iTregs and enhances the production of IFNγ under TH1.

We further demonstrate that TGFβ reduces the PI3K signalling strengthrestoring the production of cytokines under different conditions, altered by the higher PI3Ks pathway. Mice that over express PI3Ks, do not develop any signs of EAE and aretotally resistant to induced colitis transfer model on RAG1 KO model; the level of GM-CSF and IL-17A is not affected but the production of IFN$\gamma$and IL-22 is dramatically enhanced.

Using neutralizing antibodies for IFNγ and IL-22 we proved that either EAE and colitis can be both rescued in the transgenic model to a comparable level of the WT.

Conclusions: Here we show the master role of PI3Ks on $CD4^+$ T helper cells commitment; the major role of this cascade in influencing the cytokines production.

An high activity of PI3Ks leads to a TH1 outcome, a middle activity to TH17 anda low activity to Tregs.

The high production of IL-22 and IFNγ protect the mouse from EAE and colitis unveiling a complete new role of PI3Ks cascade on induction of tolerance and identify IL-22 as a new potential target in autoimmunity

P0532

The transcription factor Blimp-1 is a critical regulator of IL-10 expression by T helper 1 cells

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Purpose/Objective: Expression of the anti-inflammatory cytokine IL-10 by pro-inflammatory T helper 1 cells (Th1) is a crucial mechanism for Th1 self-regulation and the containment of inflammatory immune responses. To identify potential transcription factors that are involved in the regulation of IL-10, we highly purified IL-10 secreting and non-secreting Th1 cells and screened their gene expression profile for new candidates.

Materials and methods: Sorted murine naïve CD4 T cells were activated *in vitro* under Th1 polarizing conditions and subjected to cytokine secretion assay on day 5 following re-stimulation with PMA/Iono. Subsequently, IL-10 secreting and non-secreting T cells were highly purified via FACS sorting and analysed using an Affymetrix GeneChip® array for the expression of candidate transcription factors.

Results: We found that Blimp-1 was selectively over-expressed by IL-10 producing Th1 cells. Blimp-1 deficiency in *in vitro* as well as *in vivo* differentiated Th1 cells completely abolished IL-10 expression. In turn ectopic expression of Blimp-1 strongly induced IL-10 production by Th1 cells. Importantly over-expression of c-Maf, which also segregated with IL-10 secretion in our screen, could not rescue the IL-10 deficiency in Blimp-$1^{-/-}$ Th1 cells, although c-Maf over-expression was sufficient to induce IL-10 as well as Blimp-1 expression in wild-type T cells. Furthermore c-Maf expression levels were not altered in Blimp-1 deficient Th1 cells as well as after Blimp-1 over-expression, suggesting that Blimp-1 rather than c-Maf limits IL-10 expression. In addition, stability studies with sorted IL-10 producing Th1 cells revealed that also IL-10 re-expression highly correlated with Blimp-1 expression in contrast to c-Maf.

Conclusions: Taken together our data identify Blimp-1 as a critical transcription factor for IL-10 expression by Th1 cells and introduce an attractive target for therapeutic manipulation of this pivotal immunoregulatory mechanism.

P0533

Toll-like receptor 9 ligand promotes vaccine potency by enhancing Follicular Helper T cell development

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Purpose/Objective: Protein sub-unit vaccines promote long-term immunity through the production of high-affinity B cell memory. To be effective, vaccine priming must induce antigen-specific Follicular Helper T cells (Tfh) that control plasma cell production and memory B cell development in secondary lymphoid tissue. Thus, understanding the mechanisms of Tfh cells and how to manipulate them could have a lot of impact for vaccine design.

It was shown that soluble TLR9 ligand can be used as adjuvant for a protein antigen *in vivo* by enhancing the T-dependent antibody response. This effect require TLR signaling in dendritic cells (DC). Thus, it is clear that TLR9 ligand can promote antibody responses, there is little understanding of the rules governing how TLR9 signaling contribute to antibody responses *in vivo*. Moreover, whether complementation ofexisting vaccine with TLR9 ligand enhances adjuvancity is still unknown.

Materials and methods: We took advantage of the well-characterized T and B immune responses of C57BL/6 mice to I-Eα protein and its immunodominant MHC class II epitope using Eα52-68-I-Ab pMHCII tetramers and Flow cytometry approaches to monitor the dynamics of antigen-specific B and T cell resposnes after protein vaccination. Furthermore, using this antigen model, we can also track antigen presentation and the nature of presenting DC that appear in draining lymph nodes from immunized animals. Using this protein vaccination, we investigated the impact of CpG addition in the adjuvanticity and on antigen-specific immune response.

Results: We show that addition to vaccine adjuvant of TLR9 ligand, CpG, enhances the antigen-specific B cell responses after protein vaccination *in vivo*. In particular, this effect correlates with an increase of antigen-specific Tfh cell. Furthermore, we observed that cDC recruited in the draining LN in CpG-primed condition secrete more IL-6, a cytokine necessary for Tfh cell differentiation *in vivo*, and are more prone to induce Tfh cell differentiation directly *ex vivo* when compared to DC from primed animals without CpG. In contrast, conventional DC bearing pMHCII on their surface which are recruited in the draining LN are less abundant in CpG-primed condition. In addition, we also showed that pDC do not capture and present Ag but participate in this bias towards Tfh cell differentiation by secreting IL-6.

Conclusions: Our results suggest that TLR9 ligand through DC imprints the specialized program of antigen-specific effector Tfh function needed to promote high affinity B cell immunity *in vivo* and could serve as a basis for manipulating the formulation of the next generation of protein vaccines.

P0534

Towards identification of gene regulatory network resulting in human TH17 differentiation

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Purpose/Objective: The aim of our study is to identify key regulatory networks of the early human Th17 cell differentiation process. This will be achieved through combination of genome wide and computational methods exploiting the strategy we recently used to report fundamentally new insights into human Th2 cell differentiation (Elo et al., Immunity, 2010).

Materials and methods: $CD4^+$ T cells isolated from human umbilical cord blood were used to generate Th17 cells. To generate Th17 cells, cells were activated via cross linking T cell receptor (anti-$CD3^+$ anti-CD28) and polarization was stimulated with combination of TGF- β, IL-6, IL1β, anti-IL4, and anti-IFN-γ. Polarization of Th17 cell differentiation was confirmed by measuring the expression of several Th17 specific genes such as IL17A, IL17F, RORC, and CCR6. For transcriptional profiling, samples were collected at 0, 0.5, 1, 2, 4, 6, 12, 24, 48, and 72 h time points of culture. Total RNA was processed and hybridized on Illumina Sentrix HumanHT-12 Expression BeadChip, version 3.The microarray data analysis was done using Bioconductor package beadarray.Expression of selected genes was validated by RT-PCR, western blot, and FACS analysis.

Results: We, for the first time, report genome-wide gene expression profiling during early stages of human Th17 cell differentiation. We observed that gene expression pattern at the very early stage of human Th17 differentiation is highly dynamic. Further, we validated the selected genes at the protein level and analyzed their expression in Th1,

Th2 and iTreg T helper subsets.We found that ATP1B1, CXCR5, KDSR, and IL2RB are selectively regulated in Th17 condition during the early priming towards Th17 phenotype. On the other hand, CD52, VDR and CTSL1 were highly expressed also in response to initiation of some of the other T helper programmes. Our study provides an overview of genes and pathways regulated in response to induction of Th17 differentiation in humans and identifies several candidates which potentially play role in modulation of Th17 responses.
Conclusions: This is the first transcriptional profiling study on early Th17 differentiation process, provides the starting point for constructing the gene regulatory network and identifying new candidates possibly regulating the Th17 differentiation in human.

P0535

Upstream stimulating factors mediated regulation of RORgammaT expression in human Th17 lymphocytes

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Purpose/Objective: One of the two alternative products of RORC gene is RORγT, the orphan nuclear receptor that regulates the development of Th17 cells.Increased expression of RORγT is a hallmark of Th17 differentiation. We investigated the transcriptional regulation of RORγT in human lymphocytes to gain further insights into the process of Th17 differentiation.

Materials and methods: RORγT promoter analysis was performed with luciferase based constructs in Jurkat, HeLaand HepG2 cells.Screening for transcription factors regulating RORγT was performed by cotransfection ofRORγT promoter reporter constructs and expression vectors coding transcription factors into HeLa cells.Chromatin immunopreciptation and electrophoretic mobility shift assays were performed employing Jurkat T cells. Gene expression was assessed with real time PCR. Th17 cells were obtained from naïve $CD4^+$ T cells isolated from PBMCs cultured for 5 days under Th17 polarizing conditions. Concentration of IL-17 in supernatants was determined by ELISA.siRNA experiments were performed using nucleofection of lymphocytes with duplexes of Stealth siRNA. Statistical analysis was performed using one-way ANOVA, followed by Tukey's *post hoc* test.
Results: While nonlymphatic human cells exclusively expressed RORγ, Jurkat lymphocytes predominantly expressed RORγT. Analysis of human RORγT promoter activity with 5' deletion and *in situ* mutagenesis analysis, chromatin immunoprecipitation and overexpression of selected transcription factors, revealed that USF-1 and USF-2 are critical for RORγT expression in Jurkat lymphocytes. USFs expression was upregulated upon differentiation of Th17 cells *in vitro* and siRNA mediated knockout of USFs expression resulted in significant decrease in expression of RORγT in Jurkat and in Th17 cells.
Conclusions: We demonstrate the role of the USF-1 and USF-2 transcription factors in regulating the expression of RORγT in human lymphocytes.Thus, USFs are important for the molecular mechanisms of Th17 differentiation, and possible changes in the expression of USFs might be of interest for inflammatory conditions with a Th17 component.

Poster Session: Evolution and Selection

P0536
Evaluation of genetic diversity of IGHG in 11 Lepus species

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Purpose/Objective: The European rabbit is unique in several immunological aspects, one of which being the possession of only one IGHG. Rabbit IgG has been extensively studied and its allelic variation characterized in detail since the early days of immunogenetics. However, the information available for this molecule in the Leporids is scarce, being limited to a few species hinge and CH2 sequences. In this study we sequenced the complete *IGHG* exons for 11 *Lepus* species. (382).
Materials and methods: The four IgG exons, CH1, hinge region, CH2 and CH3, were PCR amplified and sequenced for 11 extant Lepus species: L. americanus, L. californicus, L. callotis, L. capensis, L. castroviejoi, L. corsicanus, L. europaeus, L. granatensis, L. timidus, L. townsendii and L. saxatilis. Six European rabbits were also sequenced. The primers were designed on European rabbit IGHG available sequences. (332).
Results: 32 specific nucleotide differences were observed between European rabbit and hares (Lepus sp.) IgG genes corresponding to 19 aminoacid modifications. Within hares 50 SNP were found, that translate to 26 aminoacid changes. Specific aminoacids were detected for *L. americanus, L. callotis, L. californicus, L. capensis, L. europaeus, L. saxatillis and L. townsendii*. The greatest nucleotide and aminoacidic variability is found on the CH2 and CH3 domain. No variability was found among the studied 11 hare species hinge region.
Conclusions: The studied hare species share a great sequence homology for IGHG. However, specific residues were observed for 7 *Lepus* species, mostly on the CH2 and CH3 domain but also on CH1. These could be related to resistance against specific pathogens. The hinge region is highly variable among species and IgG subclasses. Surprisingly, all 11 *Lepus* species studied share the same genetic hinge, suggesting that some selective pressure is maintaining the hinge motif in the *Lepus* taxon.

P0537
Signatures of positive selection in mammalian interleukins

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Purpose/Objective: Due to the host-pathogen co-evolution, the immune system and its genes are constantly evolving being under pressure and selection for adaptation, where advantageous mutations are highly favoured and deleterious mutations are quickly eliminated. Interleukins (ILs) have been identified as some of the genes of the immune system under positive selection in different mammals. Nevertheless, most studies do not specify the selected codons. Thus, we have searched for signatures of positive selection in mammalian ILs by using different codon-based maximum-likelihood (ML) approaches.
Materials and methods: Sequences of mammalian ILs used in the analyses were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/), Ensembl (http://useast.ensembl.org/index.html) and UniProt (http://www.uniprot.org/). Each interleukin was aligned using ClustalW implemented in the software program BioEdit version 7.1.3 and adjusted manually. To detect signatures of positive selection in individual codons of mammalian ILs, d_N/d_S ratios were compared using two ML frameworks, the HyPhy package implemented in the Data Monkey Web Server (http://www.datamonkey.org/) and CODEML implemented in PAML version 4.
Results: Signatures of positive selection were found in IL1A and B, IL2, IL4-IL10, IL12A and B, IL14-IL17A and C, IL18, IL20-IL22, IL25, IL26, IL27B, IL31, IL34, IL36A and G. Codons under positive selection identified by, at least, two ML methods varied between 1 and 15, being IL6 the interleukin with higher percentage of positive selected sites (2.28%, 15 codons) followed by IL7 (2.04%, 11 codons). No codons were detected in IL13, 17B and F, IL19, IL23, IL24, IL27A and IL29.
Conclusions: Through protein-coding sequences comparison we are able to identify proteins under positive selection. Several studies have shown that genes related with immunity are among those proteins, including ILs. In ILs, codons located in regions where these proteins interact with their receptors are prone to be positively selected in order to influence signalling intensity. Also, signatures of positive selection may be related with escaping mechanisms from antagonistic parasite-encoded proteins to maintain the ILs functions in the immune response.

Poster Session: Ion Channels

P0538

Altered calcium signalling in B and T cells from Systemic Lupus Erythematosus patients is related to STIM1 expression

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Purpose/Objective: The *calcium* sensor *STIM1* in lymphocytes is an essential mediator of the calcium influx that acts as a second messenger in response to activation and proliferation. Although mutations in STIM1 cause complex immunodeficiencies, the role of STIM1 in the T and B cell autoreactivity process during autoimmune diseases is currently unknown.

Materials and methods: Using thapsigargin (Tg), an inhibitor of calcium pump of the endoplasmic reticulum (ER), elevations of cytosolic calcium from the ER and activation of the store-operated calcium entry (SOCE) were evaluated in T and B cells from patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), primary Sjögren's syndrome (pSS), and healthy controls (HC). Concomitantly, STIM1 expression was evaluated by flow cytometry in T and B cell subsets.

Results: Higher constitutive calcium entry and increased Tg-mediated extracellular calcium entry, but not ER calcium entry, were observed in T and B cells from SLE patients compared with controls. No significant differences could be observed in SOCE/ORAI molecules by RT-qPCR. However, STIM1 expression was quantitatively different according to the disease. SLE T cells express more STIM1 than HC and pSS patients, whilst RA T cells express lower level. SLE B cells express higher STIM1 molecules than pSS B cells, whereas HC and RA B cells express similar low levels. In SLE B cells, STIM1 is overexpressed in all B cell subpopulations with highest levels detected in autoreactive transitional B cells (MFI 9.5 ± 2.3 in SLE versus 1.5 ± 0.7 in HC, $P > 0.001$). Interestingly, STIM1 level is strongly correlated with the constitutive calcium entry and the extracellular calcium influx (both, $P < 0.01$). In contrast, activity of the disease and autoantibody production were not correlated. Finally, CpG and anti-IgM co-stimulations are as efective as CpG and anti-CD40 stimulations to induce STIM1 expression in normal B cells as well as cell cycle progression in a B cell line.

Conclusions: In conclusion, these findings suggest that differential STIM1 expression may be an important determinant in the SLE autoreactivity process.

Poster Session: Lymphocyte Development

P0539
A time resolved analysis of nc/mRNA and protein expression throughout Th cell activation

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Purpose/Objective: Activation and differentiation of immune cells are crucial for an efficient and controlled immune response. To be able to secrete cytokines, naïve T helper-cells need to get in contact with antigen presenting cells (APC) to become activated and differentiate into the different T helper-cell subsets responsible for the different cytokines.These processes are controlled by a combination of transcriptional and translational regulatory mechanisms. In terms of transcriptional control non coding RNAs have been proven recently to be crucial first for epigenetic effects but also for transcription and translation. The effects can be assessed first by RNA seq and second by proteomic approaches to control for the effects of regulatory processes. Therefore we combined here RNAseq and proteomics to reveal the regulatory and effective mechanisms in T cell activation and differentiation into Th1 cells.
Materials and methods: For analysis of RNAs cells were lysed in Trizol and RNA was extracted out of the aqueous phase followed by tiling arrays and transcriptome sequencing. Proteins were extracted from the phenolic phase of the Trizol extraction and analysed via GeLC-MS/MS.
Results: We identified ~22.000 transcriptionally active regions (TARs) in the genome which are differentially expressed throughout this process and fall into diverse coding and non-coding transcript categories.Out of 10 buffy coats 2445 proteins were unambiguously identified in at least five of 10 buffy coats. Among the 409 proteins regulated over the activation time course were known transcription factors like STAT1, NF-kB and others, which are known to be involved in cellular differentiation of immune cells.
Conclusions: The combination of transcriptome and proteome data allow identification of new ncRNAs as well as deciphering the influence of non-coding sequences on the abundance of relevant factors in T cell activation.

P0540
CD8aa TCRab intraepithelial lymphocytes: a unique distinct T cell lineage

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Purpose/Objective: We aimed to define the thymic development and MHC restriction of CD8aa TCRab intraepithelial lymphocytes (IEL) of the small intestine.
Materials and methods: We cloned TCR genes isolated from naturally arising CD8aa TCRab IEL and retrovirally express these TCRs in bone marrow (BM) chimera.
Results: First, we successfully sequenced and cloned three TCRab isolated from single cells and then expressed them retrovirally in Rag KO BM chimera. Analysis of the chimera showed that all three TCR clones gave rise to T cells. The T cell that developed were CD4- CD8b- or CD8aa TCRab cells and phenotypically identical to CD8aa TCRab IEL isolated from un-manipulated wild type mice. In addition, they were preferentially found in the gut. In order to define the MHC restriction of these particular TCRs, BM chimeras in various MHC-deficient backgrounds were generated. Subsequent analysis of the chimera for the presence of CD8aa TCRab IEL demonstrated that they are dependent either on KbDb MHC I or on b2m-dependent MHC I molecules aside from KbDb.
Conclusions: Our results show for the first time that TCRs originally cloned from CD8aa TCRab IELs can only give rise to this identical T cell lineage but not conventional CD4 and CD8ab T cells. In addition, this implies that the CDR3 from the TCR sequence will exclusively allow the selection of this unique T cell population most likely through an instructive model. Finally, CD8aa TCRab IELs can also harbor clones selected on different MHC molecules.

P0541
Characterization of the development and function of thymic Hassall's corpuscles

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Purpose/Objective: Thymic Hassall's corpuscles (HCs) are enigmatic tissue structures with poorly defined origin and function. Since recent studies have indicated a role for the Autoimmune Regulator (Aire) in HC development, the aim of this study was to clarify whether HCs are derived from the $Aire^+$ medullary thymic epithelial cells (mTECs), and whether the unique features of $Aire^+$ mTECs to express and present tissue specific antigens (TSA) to the developing thymocytes, are preserved at the HC-stage of mTEC development.
Materials and methods: Two different Aire-reporter mouse models (Aire-LacZ and Aire-GFP) as well as Aire KO mice were used to elucidate the relations between Aire and HCs. Also, FACS-based cell sorting followed by quantitative PCR was used in order to define gene expression profiles of different mTEC populations.
Results: In the Aire-reporter mice the majority of HCs stained also positive for LacZ or GFP whereas in the Aire KO mice the numbers of HCs were severely reduced. The post-Aire developmental stages of mTEC development were characterized by pronounced downregulation of MHC class II and CD80, and of most of the Aire-dependent and Aire-independent TSAs, with the exception of keratinocyte-specific genes. In the final stage of maturation, the mTECs lost their nuclei to become HCs and specifically expressed certain keratinocyte-specific autoantigens, such as desmogleins (DGs) 1 and 3.
Conclusions: HCs comprise a post-Aire mTEC population which specifically expresses keratinocyte-specific antigens. Via cross-presentation of these TSAs by APCs to the developing thymocytes, HCs may contribute to tolerance induction against these pemphigus vulgaris-related antigens.

P0542
Contribution of secondary immunoglobulin heavy chain rearrangements to primary antibody diversification

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Purpose/Objective: V(D) J recombination is a process catalyzed by Recombination Activation Genes-1 and -2 in B-lymphocyte progenitors to assemble the variable (V) region genes of the immunoglobulin heavy and light chain polypeptides. In mammals, the establishment of

a sufficiently complex primary Ig repertoire is essential for life-long immunity against foreign pathogens. Current knowledge indicates that IgH V-gene diversification results from single successful VDJ recombination events. Recent reports have challenged this model showing occurrence both in humans and mice of secondary IgH rearrangements. Importantly, this process has been so far described in transgenic mouse models expressing non-functional IgH chains or in the context of autoimmunity, raising the question of its physiological relevance. This project thus aims to precisely estimate the contribution of secondary IgH rearrangements to the establishment of a normal antibody repertoire.

Materials and methods: A unique mouse strain generated by nuclear reprogramming of an intestinal IgA plasma cell expressing a non-autoreactive BCR was employed, which allows a rapid and easy tracking of B-cells undergoing secondary IgH rearrangements.

Results: B-cell progenitors in cloned IgA mice are expected to express the same pre-rearranged IgH V gene. By phenotype analysis we observed that over 20% of peripheral mature B-cells in IgA heterozygous mice expressed IgM on the cell surface, suggesting the occurrence of secondary IgH rearrangements in a substantial number of developing B cells. Sequencing of IgA rearrangements in sorted IgM^+ B cells of $IgA^{H/+}$ mice revealed that both V_H replacement and direct V_H-to-J_H joining contributed to the disruption of the original V_H rearrangement, thereby triggering novel rearrangements on the second (germline) IgH chromosome. Sequence analysis of CDR3 regions suggested that secondary IgH rearrangements likely occurred in pro-B cells as they carried *n* nucleotides introduced by terminal deoxynucleotidyl transferase (TdT). Interestingly, V-gene analysis in B cells undergoing secondary IgH rearrangements, revealed a strong bias for V-genes adjacent to the pre-rearranged V_H gene as recombination substrates.

Conclusions: Altogether these results provide evidence for a major contribution of secondary IgH rearrangements to the diversification of the primary antibody repertoire under conditions of normal B-cell development.

P0543

Differential induction of TH1-17 and TH-9 cytokines in human naïve t helper cells by B7h- and B7.1-mediated costimulation

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Purpose/Objective: ICOS and CD28 are expressed by T cells and are involved in costimulation of cytokine production in T helper (TH) cells. ICOS binds B7h expressed by several cell types, whereas CD28 binds B7.1 and B7.2 expressed by antigen presenting cells (APC). This work compared the activity of recombinant B7h-Fc and B7.1-Fc in induction of TH17 and TH9 cytokine secretion in human naïve TH cells.

Materials and methods: Purified naïve TH cells were activated with anti-CD3 mAb in the presence of either B7h-Fc or B7.1-Fc plus different combinations of rIL-1β, rIL-6, rIL-21, rIL-23, rTGF-b1, and rIL-4. Secretion of IL-17A, IL-17F, IL-17A/F, IL-10, and IL-9 was assessed by ELISA in the supernatants. Levels of the IL-26 mRNA were evaluated by Real-time PCR in the cell lysate.

Results: Results showed that, in the presence of $rTGF\text{-}b1^+$ rIL-1b (a TH17 polarizing condition), B7h-Fc was more effective than B7.1-Fc in inducing IL-17A and IL-10, whereas B7.1-Fc was more effective in inducing IL-17F and IL-26. These different patterns were supported by further addition of rIL-6, rIL-21, and rIL-23 in the culture medium. In the presence of $rTGF\text{-}b1^+$ rIL-4 (a TH9 polarizing condition), B7.1-Fc induced high levels of IL-9 that was instead barely detectable using B7h-Fc. Upon dual costimulation with both B7h-Fc and B7.1-Fc, secretion of IL-17A and IL-17F was similar to that induced by B7.1 alone, with predominance of IL-17F, but secretion of high levels of IL-10 mimicked that induced by B7h-Fc alone; secretion of IL-9 displayed intermediate levels between those induced by B7.1 alone and B7h-Fc alone.

Conclusions: These data showed that B7h and B7.1 have different effects on secretion of TH17 and TH9 cytokines in naïve TH cells, and that the effect of contemporary use of both costimuli is not the simple sum of the effects exerted by each of them.

P0544

Flipping of lipids by a putative transporter is essential for B-cell development and function

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Purpose/Objective: B cells with their ability to secrete a wide array of antibodies against various invading pathogens play a central role in the generation of humoral immunity. The development of B cells takes place in the bone marrow of most mammals in a highly regulated process that depends on cell intrinsic and extrinsic factors such as signaling through the precursor-B cell receptor (pre-BCR) and, at least in the mouse, the Interleukin-7 receptor (IL-7R), and the activation of transcription factors. However, the role of the composition of the cell membranes on the development of B cells is still poorly understood. Here we describe a new ENU-induced X-linked B cell deficiency syndrome in mice caused by a mutation in *Atp11c*, a previously uncharacterized member of the P4-ATPase family thought to serve as flippases that catalyze aminophospholipid transport from the exoplasmic to the cytoplasmic leaflet of cell membranes.

Materials and methods: Analysis of mice with an ENU-induced point mutation in *Atp11c* by flow cytometry and genetic crosses as well as *in vitro* assays to determine the uptake of aminophospholipids in cells from mutant and control animals.

Results: We show that ATP11C deficient mice have a near complete absence of B cells in the blood due to a severe block at the transition from the pro-B to pre-B cell stage of B cell development in the bone marrow. This block could partially be rescued by the introduction of a transgenic B cell receptor whereas elevated levels of Interleukin-7 or enforced expression of Bcl-2 showed only a minimal increase in peripheral B cell numbers. Similarly, in the spleen the number of B cell was also greatly reduced except for normal numbers of marginal zone B cells. This correlated with a reduced internalization of fluorescently labeled phosphatidylserine in developing pro-B cells but not in other developmental stages of B cells or different cell types in the bone marrow and spleen. Interestingly, despite the reduced B cell numbers the response to T-independent antigen was normal but both the primary and memory antibody response to T-dependent antigens was greatly reduced with the most severe reduction seen in the germinal centre-dependent response to the hapten azo-benzen-arsonate.

Conclusions: The results demonstrate that the phospholipid transporter ATP11C is crucial for early B cell development and antibody production, and reveal an intimate and novel connection between phospholipid transport and B lymphocyte development and function. Our results also provide a new candidate gene for the many currently unclassified human primary immune deficiencies.

P0545

HDAC7 plays a significant role in IL7 signalling during the Pro-B/Pre-B transition of B cell development

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Purpose/Objective: In many cellular systems Histone Deacetylases (HDACs) play important roles in the regulation of cell differentiation, proliferation and survival. HDACs regulate chromatin structure and thus gene transcription by reversing histone lysine acetylation. As such they been identified as key players in the development of the immune system with evidence suggesting HDAC7 acts as a gene expression switch regulating T cell development and function while HDAC9 increases regulatory T cell function by interacting with the transcription factor FOXP3. A role for HDACs in B cell development and function is poorly characterised and where our interest lies.
Materials and methods: We have bred an HDAC7 floxed/floxed mouse with a *vav*-cre transgenic mouse in order to delete HDAC7 from all haematopoietic cells including B cells. To investigate a role for HDAC7 in B cell development we analysed the phenotype of lymphoid tissues in WT and KO mice (Spleen, Lymph Nodes and Thymus) using flow cytometry and qPCR.
Results: Using flow cytometry we identified a significant reduction in size of these tissues due to a large reduction in cellular content of B cells and T cells. To investigate the B cell phenotype further we looked in the bone marrow again identifying a significant reduction of Pre-B, immature and mature B cells. A small but significant increase in Pro-B cell number suggested a partial block in development between the Pro-B and Pre-B stage of development. This is a critical point during B Cell development where IL7 signalling drives expression of the pre-BCR complex. The pre-BCR complex in turn induces specific intracellular signals that drive the transcription of genes relevant for the proliferation, survival and expansion of developing B cells. In addition, defects in the expression of IL-7Ra chain in HDAC7$^{-/-}$ pro-B cells, as well as down stream effects such as RAG1/2 expression and IgM expression may account for the observed B cell phenotype in our mice.
Conclusions: These data suggest a key role for HDAC7 in controlling B cell development and further highlights the importance of HDACs within the adaptive immune system.

P0546

IL-7Ra plays a critical role in the development of B cells

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Purpose/Objective: IL-7 is a critical cytokine throughout lymphocyte biology and controls the development of T and B cells from lymphocyte precursors in the thymus and bone marrow. The molecular signalling events downstream of the IL-7Rα are not fully characterised. Y449 is critical for the development and function of T cells; the role of this motif has not been investigated in B cells, and due to the clear role of IL-7Rα in B cell development, we decided to evaluate the role of IL-7Rα^{Y449} motif in B cell development.
Materials and methods: We used flow cytometry to examine a knock-in mouse possessing a point tyrosine to phenylalanine mutation at position 449 (IL7Rα^{449F} mice) within the cytoplasmic YxxM SH2-binding motif of IL-7Rα, as well as IL-7 over-expressing, TSLPR$^{-/-}$ and IL-7Rα$^{-/-}$ mice.
Results: Signalling downstream of IL-7Rα^{Y449} is critical during early B cell development, as numbers of B cells in the bone marrow of IL-7Ra$^{-/-}$ mice and IL-7Ra^{449F} were reduced at pro-B and pre-B cell stages, suggesting that Y449 is the major signalling residue downstream of the receptor in bone-marrow B cell development. IL-7Rα also controls B cell development in the spleen, as IL-7Rα^{449F} mice have a partial and IL-7Rα$^{-/-}$ mice a near-total block at T1-T2 transition. However, IL-7Rα^{449F} was required for follicular, but not marginal zone B cell populations. Over-expression of IL-7 had the opposite effect on follicular B cells, increasing their numbers, but had no effect on marginal zone B cells. Any role for TSLP was ruled out, as TSLPR$^{-/-}$mice had an identical B cell phenotype to wild-type mice. Experiments using bone-marrow chimera suggested that the defect seen in pre-B cells was due to defective IL-7Rα signalling within the B cell, whereas the splenic defect was due to defective IL-7Rα signalling in non-B cells, as WT and IL-7Rα^{449F} B cells showed an identical phenotype in a mixed environment and did not express IL-7Ra nor signal through pSTAT5 in response to IL-7.
Conclusions: IL-7 plays a key role in the development of B cells from the earliest stages, however signalling downstream of IL-7Rα Y449 is only required at the pro-B and pre-B cell stages in the bone marrow. However, in the spleen, follicular, but not marginal zone B cells are critically dependent on IL-7Rα signalling levels and are controlled by IL-7Ra signalling in an as-yet unidentified manner extrinsic to the B cell compartment.

P0547

Inhibition of glycogen synthase kinase-3 regulates T cell development *in vitro*

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Purpose/Objective: The development of functional non-autoreactive T cells requires receptor-mediated transition through multiple checkpoints in the thymus. Double negative 3 (DN3) thymocytes are selected for the presence of a rearranged TCR beta chain in a process termed beta-selection which requires signalling via the preTCR and Notch1. Pre-selection DN3 are referred to as DN3a and express low levels of CD27 and CD98, while post selection DN3 (which are TCRbeta positive) express higher levels of CD27 and CD98. Additional signalling from CXCL12 ensures optimal proliferative expansion of DN3 thymocytes. Signal integration by these receptors converges on core pathways such as the Phosphatidylinositol-3-kinase (PI3K) pathway. Glycogen Synthase Kinase 3 (GSK3) is generally thought to be negatively regulated by the PI3K pathways.
Materials and methods: Murine DN3a were FACS sorted as negative for CD4/CD8/CD44/CD11b/CD19/NK1.1/Gr-1/$\gamma\delta$TCR/Ter119 and CD25highCD98low. FACS sorted DN3a were cultured with OP9 or OP9-Delta Like 1 stromal cells or in wells that had been coated with 10 μg/ml of recombinant mouse Delta Like 4. In the stromal cell-free cultures DN3a cells were cultured in the presence or absence of recombinant murine CXCL12 and IL-7. In some experiments the GSK3 inhibitor CHIR99021 was added with the DN3a cells from the start of culture. After up to 96 h of culture numbers of gated lymphocytes were determined and analysed by FACS.
Results: We have shown that a GSK3-inhibiting drug, CHIR9902,1 promotes the proliferative expansion of DN3a cultured with recombinant Delta Like 4 and CXCL12. Here we show that developmental progression of DN3a is promoted by CHIR99021. Furthermore, inclusion of CHIR99021 allowed differentiation in the absence of preTCR- or Notch1-mediated signalling. Inactivation of GSK3 using CHIR99021 appears to antagonize IL-7-mediated inhibition of development at the DN stage. In addition to the effect on T cell development, CHIR99021 increased IL-7 dependent proliferation and caused enhanced cell recovery in these experiments.
Conclusions: These experiments indicate a potentially important role for inactivation of GSK3 during the process of beta-selection. A stromal free culture system that promotes beta-selection may offer a

new drug discovery platform for screening regulators of proliferation, differentiation and apoptosis.

P0548

Matrix attachment regions flanking the IgH intronic Eμ enhancer are important cis- and trans-regulators of somatic hypermutations

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Purpose/Objective: The immunoglobulin heavy chain (IgH) intronic enhancer region (Eμ) is a combination of both an enhancer core element (220 bp) and two 310−350-bp flanking scaffold/matrix attachment regions (S/MARs herein named $MARs_{E\mu}$) that were first defined by nuclear matrix-binding assays *in vitro*. The Eμ core enhancer region is critical for efficient V-D-J recombination at early stages of B cell development. Strikingly, the physiological role of $MARs_{E\mu}$ is still unclear even if several nuclear factors have been found to bind to this region: a B cell transcription factor named BRIGHT/Arid3a; AT rich binding proteins expressed in T cells and in B cell progenitors (SATB2); and also a negative regulatory factor named NF-μNR whose expression decreases at late stages of B cell development when cells express high levels of Ig heavy chains.
Materials and methods: We generated a mouse model carrying an endogenous deletion of MARs and performed a detailed analysis of its consequences on B cell development.
Results: We found that, unlike the core Eμ element, the absence of MARs did not affect B cell development but led to a significant decrease in somatic hypermutations in Peyer's patch germinal centre B cells. In the intronic region downstream IgH J segments, the mutation frequency was also significantly reduced in the corresponding region of the Ig Kappa locus.
Conclusions: Our data revealed that IgH $MARs_{E\mu}$ regions are critical regulatory elements that participate in efficient recruitment of SHM machinery to Ig loci target sequences. In germinal centre B cells, we postulate that $MARs_{E\mu}$ also act *in trans* to recruit Ig Kappa loci and contribute spatially to the SHM process.

P0549

Progenitor deprivation provokes acute T cell lymphoblastic leukemia

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Purpose/Objective: Continuous thymus function is thought to depend on a steady supply of T cell progenitors from the bone marrow. To address the fate of a thymus in the complete absence of developmentally competent bone marrow progenitors, we have transplanted normal wild-type thymus into *Rag2$^{-/-}$gc$^{-/-}$KitW/Wv* mice.
Materials and methods: Compound mutant *Rag2$^{-/-}$gc$^{-/-}$KitW/Wv* mice lack competitive hematopoietic stem cells (HSC) and are devoid of T cell progenitors. Using this strain as recipient for wild-type thymus grafts,.
Results: we unexpectedly find that thymocytes persist and that T lymphocytes continue to be produced and exported for several months. Sequencing of the expressed alpha and beta TCR loci in progenitor-deprived thymus grafts shows that the TCR repertoire is still diverse. The analyses of single mutants as recipients for the thymus transplants indicate that *gc*-mediated signals alone play a key role in the competition between thymus-resident and bone marrow-derived progenitors. Hence, the turnover of each generation of thymocytes is not only based on short life span but is also driven via expulsion of resident thymocytes by fresh progenitors entering the thymus.However, a frequent consequence of progenitor-independent thymus autonomous function is the development of T cell acute lymphoblastic leukemia (T-ALL).Recipient mice developed hepatosplenomegaly and full-blown signs of T-ALL. Blast cells in bone marrow, blood and spleen had cell surface phenotypes reminiscent of T-ALL in humans, the tumors were transplantable and were clonal, based on the analysis of the TCR rearrangements. These murine T-ALLs bore mutations in the heterodimerization and PEST domains of *Notch1*. *Rag1* and *Rag2* mRNA continued to be expressed in the leukemic cells but Rag expression was not required for leukemogenesis.
Conclusions: We propose that competition between old and new progenitors in the thymus provides a quality control mechanism that ensures that young outcompete old progenitors, and that abrogation of this competition results in thymus-intrinsic T cell development, followed by the development of leukemia.

P0550

Spatio-temporal regulation of Notch ligand expression defines functional microenvironments in the human thymus: implications in the TCR Alpha Beta versus TCR Gamma Delta T-cell fate decision

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Purpose/Objective: Interaction of Notch receptors with Notch ligands (DLL1, DLL4, JAG1, JAG2) expressed on the thymic microenvironment (TME) is essential to support T-cell development. DLL4 is the essential ligand that drives T-cell specification, but little is known about the ligands involved is subsequent differentiation of pre-T cells into either TCR$\alpha\beta$ or TCR$\gamma\delta$ cells. As T-cell development implies the active movement of developing thymocytes throughout distinct thymic regions, we ought to determine whether a differential distribution of Notch ligands at defined anatomical regions could endow discrete intrathymic niches with specific developmental functions once T-cell specification has been induced by DLL4.
Materials and methods: We analysed the spatio-temporal regulation of Notch ligand expression in the human thymus by immunohistochemistry and confocal microscopy. The characterization of particular TME niches defined by Notch ligands *in vivo* provided the framework to approach the specific functions of those ligands in the TCR$\alpha\beta$ versus TCR$\gamma\delta$ cell fate of human pre-T cells using the OP9 co-culture system.
Results: We found that distinct Notch ligands are confined to defined histological regions and stromal cell types, and this distribution changes along thymus ontogeny. The four ligands are expressed at high levels in the postnatal thymus (PNT) at the cortico-medullary junction (CMJ) and thymic medulla. In contrast, JAG1 and DLL4 are essentially undetectable in the PNT cortex, although DLL4 is expressed on cortical thymic epithelial cells (cTECs) in the human embryonic thymus (<19 weeks). Instead of DLL4, JAG2 is the most prominent ligand expressed by cTECs in the PNT, although low DLL1 expression levels are also found.

Functional assays show that *a*ll ligands allow the development of TCR$\alpha\beta$ and TCR$\gamma\delta$ cells. JAG2 almost exclusively triggers the generation of TCR$\gamma\delta$ cells, which however expressed an immature phenotype. In contrast, JAG1 impairs TCR$\gamma\delta$ fate, thus balancing TCR$\alpha\beta$ and TCR$\gamma\delta$ cell fates. Additionally, JAG1 drive TCR$\gamma\delta$ cells mainly to the conventional phenotype.

Conclusions: Specific TME niches are defined by Notch ligand expression during human thymopoiesis. This finding together our functional data highlight the importance that spatio-temporal regulation of Notch ligand expression may have *in vivo* to control the TCR$\alpha\beta$ versus TCR$\beta\gamma\delta$ cell fate and differentiation.

P0551
Stage- and ligand-specific regulation of dendritic cell development by Notch signalling in the human thymus

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Purpose/Objective: Early thymic progenitors (ETPs) are multipotent precursors that can generate plasmacytoid and conventional dendritic cell (pDCs and cDCs), in addition to T-cells, both *in vitro* and *in vivo*. Given that DCs are normal residents in the steady-state thymus, it is believed that at least part of thymic DCs are generated *in situ*. How ETPs escape from intrathymic signals provided by Notch ligands and avoid T-cell fate specification to generate DCs has not been established; neither the developmental relationship between the two DC thymic lineages, nor the developmental cues that determine the alternative pDC and cDC cell fates are known.
Materials and methods: To assess these questions, we used an *in vitro* DC differentiation assay, based on the co-culture of human ETPs or DC-primed intrathymic progenitors onto OP9 stromal cells expressing different Notch ligands, together with gene expression analyses.
Results: We show here that both DC subtypes are generated from human ETPs through intermediate pDC/cDC progenitors that can be identified also *in vivo*. Such thymic intermediates display phenotypic and genetic myeloid-associated features and have lost T-cell potential. According to the current view that Notch signaling induced by Delta-like-1 ligand (DLL1) binding impairs non-T cell development, we found that DLL1-induced signaling hampered the generation of intermediate pDC/cDC progenitors from ETPs. In contrast, Jagged-1 (JAG1) was permissive. However, once generated, DC progenitors were dependent on survival signals provided by either DLL1 or JAG1, which also favour their differentiation into either pDCs or cDCs. Indeed, DLL1 promoted cDC but did not affect pDC differentiation from pDC/cDC precursors, while differentiation of both DC subtypes was promoted by JAG1-mediated signaling.
Conclusions: Our results provide evidence that Notch signaling controls the development of intrathymic DCs in a stage- and ligand-specific manner and suggest that the spatial regulation of JAG1 versus DLL1 expression in the human thymus could provide specific niches supporting the final fate of ETPs and thereafter of pDC/cDC common progenitors.

P0552
TC21 provides survival signals during negative selection

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Purpose/Objective: During thymic development the TCR expressed by immature thymocytes is affinity-proofread according to its interaction with Mayor Histocompatibility Complex (MHC) loaded with self-peptides presented by thymic antigen presenting cells (APC). If affinity of the rearranging TCRs for self-peptide/MHC is too high, thymocytes enter in a program of apoptosis that eliminates potentially harmful autoreactive T cells. TC21 is a GTPase of the Ras-related (RRas) subfamily which interacts with the TCR and is important for tonic TCR signalling and survival of mature T cells. The aim of this work was to study the role of TC21 during thymic development.
Materials and methods: We used C57BL/6 TC21-deficient and WT mice non transgenic and transgenic for AND TCR receptor with H-$2^{k/b}$ and H-$2^{b/b}$ haplotypes. Classical and non-classical Ras GTPases expression in thymic subpopulations was measured by absolute and relative qPCR. Different markers of thymic development were studied by flow cytometry. Tunnel immunofluorescence was used to quantify apoptotic thymocytes. Activation of several signalling pathways was studied by Immunoblot upon TCR stimulation with different ligand concentrations and time-points.
Results: The absence of TC21 in AND TCR transgenic (Tg) mice expressing the I-Ek haplotype, in which the TCR-MHC interaction is of higher affinity, provokes a blockade at the DP stage with no virtually SP4 generated. These thymocytes have upregulated the expression of Nur77 and Annexin V, indicating that the DP blockade is caused by an exacerbated negative selection. Although apparently there are no differences in the phenotype of non-transgenic mice, a Tunnel assay shows more apoptotic thymocytes in TC21 KO thymuses. Furthermore, TC21-deficient AND Tg thymocytes have increased sensitivity to apoptosis induced by stimulation with antigen-loaded dendritic cells *in vitro*. In this system, TC21-deficient thymocytes show impaired phosphorylation of Akt, suggesting that TC21 is required for the activation of a TCR-dependent phosphoinositide 3-kinase (PI3K) pathway in the thymus.
Conclusions: TC21 plays an important role during negative selection by modulating TCR-induced pro-survival signals.

P0553
The impact of DNA and RNA alterations of Ikaros (IKZF1) transcription factor on protein expression and localization

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Purpose/Objective: Ikaros is a key regulator of lymphocyte development. Multiple IKZF1 isoforms arise via alternative splicing, including functional IK1, IK2 isoforms and short isoforms lacking DNA-binding capacity (IK4, IK6, IK8) that are predicted to act as inhibitors of IKZF1 function. Recently, it was shown that gene deletions are the main cause of short isoforms expression in acute lymphoblastic leukemia (ALL). In a cohort of 206 children with ALL we found 14 cases (7%) with deletions of at least one exon of IKZF1 that were not always reflected in altered gene expression. Conversely, 5/206 (2%) cases without deletion expressed Ik6 isoform. Both types of alterations predicted poor prognosis. Data on the impact of IKZF1 gene alterations on protein expression are still inconsistent.
Materials and methods: We examined IKZF1 protein expression by Western blot and localization by confocal microscopy in cases with different types of IKZF1 alterations.
Results: In sorted normal B, T cells and monocytes, IKZF1 was located in the nucleus showing a punctate pattern. Interestingly, during mitosis, IKZF1 protein was completely shifted to cytoplasm. In 7 ALL cell lines without genomic deletion preferentially expressing IK1/IK2 mRNA and protein the vast majority of protein was localized in the nucleus. In case with monoallelic DNA deletion of exon 4–7 causing Ik6 mRNA expression we only detected Ik6 mRNA while Western blot showed Ik6 together with Ik1. Protein was localized partly in the cytoplasm and partly in the nucleus. This contradicts the general

consensus on cytoplasmic localization in IK6 expressing cells.In a cell line with monoallelic deletion of exons 4−8 we detected only long isoform Ik1 that was likely to originate from the second allele. The protein was mostly present in nucleus.Surprisingly, in two cell lines with monoallelic deletion of exon 1−7 we found only Ik6 mRNA that was confirmed by Western blot although protein expression was weak. Protein was localized in the nucleus with a smaller proportion present in the cytoplasm. The mechanism of this expression pattern has still to be clarified.

Conclusions: Our data showed that the incomplete correlation between IKZF1 alterations at DNA and RNA level further increases at the protein level, which is the key determinant of IKZF1 role in malignant transformation.

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P0554

The Influence of Lck on thymic selection thresholds and peripheral T Cell activation

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Purpose/Objective: Selection of the T cell repertoire in the thymus is governed by the need to create a repertoire of peripheral T cells that can respond to any foreign antigen in the context of self-MHC, while enforcing central tolerance to self-antigens. The affinity of the selection window has been shown to be very narrow. Perturbations in signalling molecules that reduce the affinity of thymic selection can lead to the production of a peripheral repertoire with increased autoimmunity, as has been shown for mutations in the Zap-70 kinase.

$p56^{lck}$ (Lck) is the most proximal tyrosine kinase required for effective TCR triggering upon TCR engagement by peptide:MHC, upstream of Zap-70. We asked how altering the expression of Lck influenced selection of thymic T cells and activation of peripheral T cells in two mouse models in which expression of Lck is altered.

Materials and methods: Lck^{ind} mice have conditional expression of Lck under the control of a tetracycline inducible transgene. Lck^{ind} mice have high expression of Lck in the thymus but lower -10 to 20% of wild-type (WT) − expression of Lck in the periphery. Lck^{VA} mice express Lck constitutively from a T cell specific transgene and have very low expression of Lck (~5% of WT) in both the thymus and periphery. Cells were analysed by FACS and Western Blot.

Results: Peripheral Lck^{ind} T cells have higher activation thresholds, requiring approximately fivefold more antigen, than WT cells to be triggered. In contrast, Lck^{VA} mice have comparable activation thresholds to WT cells. We asked whether this difference in sensitivity of peripheral T cells from these two lines was a consequence of the amount of Lck available for thymic selection.

Lck^{VA} mice display altered TCR expression pattern in the thymus during development compared to WT and Lck^{ind}. On a TCR transgenic background Lck^{VA} mice also show lower expression of TCR on peripheral T cells.

Conclusions: The level of Lck available to T cells in the thymus influences thymic selection and subsequently affects TCR expression and the behaviour of peripheral T cells.

P0556

The role of pTalpha isoforms in alpha beta T cell development

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Purpose/Objective: Mature $\alpha\beta$ T cells, that express a heterodimeric $\alpha\beta$ T cell receptor (TCR$\alpha\beta$), develop in the thymus from a common lymphoid progenitor and are indispensable components of a healthy immune system, displaying a range of functions (being cytotoxic, regulatory, or providing 'help') and sites of migration (to tissues such as gut versus secondary lymphoid organs). Early in thymic development, in order to successfully traverse an important developmental checkpoint, immature thymocytes up-regulate a pre-TCR complex that is composed of a rearranged TCRβ chain, the invariant pTα chain, and various CD3 signalling molecules. The availability of two alternatively-spliced pTα isoforms ($pT\alpha^a$ and $pT\alpha^b$) provide the cell with the choice between two distinct preTCR complexes ($preTCR^a$ and $preTCR^b$). However, the truncated form; $pT\alpha^b$, has been frequently omitted from studies or described as non-functional despite previous work suggesting the opposite. Our recent work has demonstrated that despite lacking an extracellular Ig-loop that was previously implicated in pre-TCR signalling, $pT\alpha^b$ could successfully drive early T cell development. The aim of this study was to ascertain a role to both pTα isoforms in T cell development.

Materials and methods: BAC-transgenic animals expressing only full-length pTα ($pT\alpha^a$) were generated. Subsequently, $\alpha\beta$ T cell subsets in thymuses, lymph nodes and gut of transgenic animals and controls were analyzed by flow cytometry. Positive selection at the DP stage was investigated by staining intracellular phosphorylated ERK following cross-linking of TCR$\alpha\beta$.

Results: Preliminary data suggest that $pT\alpha^a$ alone is sufficient for the development of conventional $\alpha\beta$ T cells ($CD8^+$ and $CD4^+$ T cells), but does not seem to rescue a particular population of gut T cells referred to as unconventional $TCR\alpha\beta^{(+)}$ $CD8\alpha\alpha^{(+)}$ intraepithelial lymphocytes (IELs). Furthermore, our data show a favored development of SP thymocytes ($CD4^+$ or $CD8^+$) and different levels of phosphorylated ERK in DP thymocytes ($CD4^+$ $CD8^+$) of transgenic animals.

Conclusions: Contrary to previous belief, our work showed that $pT\alpha^a$ and $pT\alpha^b$ isoforms do not have redundant functions in $\alpha\beta$ T cells development. Additionally, the data reveal a hitherto unrecognized role for pTα in the positive selection of $\alpha\beta$ T cells at the $CD4^+$ $CD8^+$ 'DP' stage.

P0557

The role of the ileal Peyer's patches and the bone marrow in B cell development of swine

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Purpose/Objective: Pigs are traditionally categorized into group of animals that use Gut Associated Lymphoid Tissues (GALT) for generation of B cell repertoire. This group include chicken that use bursa of Fabricius, rabbit that use sacculus rotundus (or appendix), and other ungulates including swine that use Ileal Peyer's Patches (IPP). Since bursectomy results in B cell deficiency, we wondered if resection of the IPP of piglets would have a similar effect.

Materials and methods: Flow cytometry and molecular immunology methods were used to compare IPP from resected, surgical shams and untreated germ-free piglets, all of which were later colonized with a defined commensal flora. A similar approach was used for analyses of flow cytometry sorted cell populations from the bone marrow.

Results: Studies disprove categorization of swine (and probably other ungulates) into group of animals that use GALT for generation of B cell repertoire. It has been shown that IPP are not a significant source of B cells, are not required for maintenance of the systemic B cell pool and are not a site of primary B cell lymphogenesis because resection of IPP does not have any effect to development of systemic B cells. On the other hand, colonization of IPP caused a shift from the fetal type of lymphocyte distribution to the adult type that is characteristic by prevalence of mature effector lymphocytes. Comparison of germ-free

with colonized pigs proves that lymphocyte development in IPP is dependent on colonization. According to finding that IPP are a secondary lymphoid tissue, we have extended reported studies by analyses of RAG, TdT, VpreB expression and the presence of DJ_H, VDJ_H, VJ_L rearrangements and signal joint circles (SJC) in the genom of flow cytometry sorted cell populations from the bone marrow. The results clearly show that porcine B cells are developed in the bone marrow throughout entire life.

Conclusions: The results indicate that porcine IPP are not required for systemic B cell generation or maintenance, but are secondary lymphoid tissue that appear important in immune responses to colonizing bacteria. On the other hand, the bone marrow is fully capable of B cell lymphogenesis and remains active throughout entire life. This work was supported by grants P502/10/0038 and P502/12/0110 from the Czech Science Foundation, and a grant ME09089 from the MSMT.

P0558
The role of the tyrosine kinase receptor RET in T cell development and function

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Purpose/Objective: The neuronal growth factor family includes the glial cell-line derived neurotrophic factor (GDNF) ligands (GFLs), which signal through the RET tyrosine kinase receptor. Importantly, *Ret* expression in T lymphocytes has been reported both in mouse and human, and while a role for RET ligands in thymocytes has been suggested the function of RET in mature T lymphocytes remains elusive. Using cellular and genetic approaches, we are investigating the expression and function of RET in T cell development and function.

Materials and methods: Materials and methods are briefly described within the results section.

Results: Our results confirm the expression of *Ret* and of the co-receptors *Gfra1* and *Gfra2* by developing thymocytes and mature $CD4^+$ and $CD8^+$ T cells. In contrast to previously suggested, our results using *Gfra1*$^{-/-}$, *Gfra2*$^{-/-}$ and *Ret*$^{-/-}$ embryos show that embryonic T cell development is not affected. Adult T cell development was also unaffected in *Ret*$^{-/-}$ FL chimeras or in *Ret*$^{fl/fl}$ animals bred to *CD2Cre* mice, excluding a role for RET-mediated signals in T cell development. This finding allowed us to use T cells from these animals and investigate the role of RET in peripheral T cell homeostasis and function. Importantly, gene expression analysis revealed that *Ret* is developmentally regulated being absent in naïve T cells but present in activated/memory phenotype T cells, thus suggesting a role in the establishment of memory T cell pools or in the effector function of T cells. The analysis of the peripheral *Ret*$^{-/-}$ T cell pools ruled out a major role for RET-mediated signals in peripheral T cell homeostasis, as steady-state subpopulation structure was unperturbed. We have thus focused our studies on T cell effector function. We found fast up-regulation of *Ret* and co-receptors upon *in vitro* activation of T cells and we have observed an impact of RET-absence in *in vitro* polarization cultures, with higher differentiation of IL-10 producing $CD4^+$ T cells in TH2 polarization conditions. In order to translate these findings into *in vivo* settings, we have treated WT or Ret$^{fl/fl}$xCD2Cre with anti-CD3 as this treatment results in IL17-dependent gut inflammation under the control of IL10-producing regulatory T cells. Our initial results confirm our hypothesis and suggest that RET-mediated signals modulate the regulatory arm of $CD4^+$ T cell responses, resulting in diminished IL17 production in the absence of RET.

Conclusions: We conclude that RET-dependent signalling is not involved in T cell development but is most likely playing a role in IL10-dependent regulatory function of $CD4^+$ T cells.

P0560
Thymic block in T cell development in rheumatoid arthritis

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Purpose/Objective: Background. Several lines of evidence show, that the T cell compartment of RA patients is characterized by premature immunosenescence and a disturbed homeostasis. Latent cytomegalovirus (CMV) infection has been shown to contribute to homeostatic exhaustion of $CD4^+$ T cells in RA. Aim of the study was to analyze if the reduced frequency of naïve $CD4^+$ T cells in the peripheral blood of RA patients is due to a reduced bone marrow output of progenitors cells.

Materials and methods: Methods. Mononuclear cells of the peripheral blood of 33 RA patients and 33 age-matched healthy controls (median age 63 years) were analyzed by flow cytometry. RA patients were all $ACPA^+$ and RF^+, and 50% were SE-$DR4^+$. An additional healthy cohort ($n = 60$) was recruited to analyze the influence of age on various parameters.

Results: Results. The frequency of the pre-thymic lymphoid progenitor ($CD34^+$ /Lin-/CD24-/$CD10^+$) as well as the frequency of recent thymic emmigrants (RTEs, $CD4^+$ /$CD45Ra^+$/$CD31^+$) negatively correlates with age in healthy controls. Additionally, the lymphoid progenitor frequency positively correlates with the frequency of RTEs.

The frequency of the lymphoid progenitor is increased in RA patients compared to healthy controls (41 cells per 1E07 PBMC versus 28 cells per 1E07 PBMC, $P = 0.0063$). However, the thymic output, characterized by the frequency of RTEs, is decreased in RA patients (32.0% versus 44.1%, $P = 0.003$). In RA patients, the lymphoid progenitor frequency does not correlate with the frequency of RTEs.

The increased frequency of the lymphoid progenitor in RA is not influenced by CrP levels, tender/swollen joint count, the presence of SE-DR4 and DAS28 score. However, CMV Ig^+ patients have increased frequencies of the pre-thymic lymphoid progenitor compared to CMV Ig- RA patients (50 cells per 1E07 PBMC versus 32 cells per 1E07 PBMC, $P = 0.03$).

Conclusions: Conclusion. The thymic output of $CD31^+$ recent thymic emigrants is influenced by the availability of pre-thymic lymphoid progenitor cells in healthy controls. The frequency of the pre-thymic lymphoid progenitor is increased in RA compared to healthy controls, while the thymic output of $CD31^+$ RTEs is reduced. We hypothesize, therefore, that insufficient thymic T cell genesis is the underlying cause of the disturbed T cell homeostasis in this disease, and might also contribute to the pathogenesis of the disease.

P0561

TNF superfamily members have different roles in thymic epithelial microenvironment

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Purpose/Objective: Thymic epithelial cells (TECs) play a key role in the thymic selection of T-cells due to their ability to express a wide variety of tissue restricted antigens (TRAs) as well as thymic chemokines that guide immature T-cells throughout their maturation. TRAs are expressed by mature $MHCII^+CD80^+$ medullary thymic epithelial cells (mTECs), at which stage they also express Autoimmune Regulator (Aire), a transcription factor shown to be responsible for the expression of a large number of TRAs. However, the signals that drive epithelial maturation and chemokine expression remain largely unknown. Previous studies have shown that RANK-RANKL signaling is necessary for producing $Aire^+$ mature mTECs. We aimed to further study the effects of tumor necrosis factor superfamily (TNFSF) members RANKL, CD40L, LIGHT, TNF-alpha and lymphotoxin beta (LT-beta) on thymic epithelium maturation along with chemokine expression in murine fetal thymus organ culture (FTOC).

Materials and methods: Thymic lobes of embryonic day 16.5 mice were removed and cultured *ex vivo* for 6 days in the presence of 2′-deoxyguanosine. Thereafter the lymphocyte-depleted lobes were stimulated with selected TNFSF members for 48 h. Material was collected and analyzed for changes in gene expression using quantitative real-time PCR.

Results: RANKL on its own was sufficient for maintaining the expression of Aire and TRAs in *ex vivo* organ culture depleted of $CD45^+$ cells. Out of the TNFSF members tested RANKL was also the only one able to induce Aire and TRA expression. LIGHT and CD40L alone did not seem to have any effect on the expression of either TRAs or chemokines. However, RANKL and especially LT-beta and TNF-alpha potently induced the expression of several chemokines including the CCR7 ligands CCL19 and CCL21.

Conclusions: Our results demonstrate how distinct intrathymic signals are responsible for different aspects of thymic function. Only RANKL seems to regulate Aire and TRA expression while thymic chemokines share a broader selection of inducers, including TNF-alpha and LT-beta in addition to RANKL. We show that these TNFSF members work cooperatively on different aspects of creating a proper thymic microenvironment.

Poster Session: Lymphocyte Signalling Mechanisms

P0562
CD46 glycosylation controls its expression and function in human T cells

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Purpose/Objective: T cell development, differentiation and activation modulate the glycosylation profile of glycoproteins on T cells, and levels of glycosylation of several glycoproteins have been shown to control T cell responses. CD46, a complement regulator, acts as a costimulatory molecule for human T cells and induces Tr1 differentiation, a subset of regulatory T cells characterized by large amounts of IL-10 secretion. CD46 ectodomain contains 3 N-glycosylation and multiple O-glycosylation sites. Proteolytic cleavage of CD46 occurs upon T cell activation and has been shown to be important to regulate T cell activation and cytokine production. Herein, we determined whether CD46 glycosylation was important for its processing and function.
Materials and methods: $CD4^+$ T cells, purified from blood, were first activated by anti-CD3/CD46 antibodies in presence or absence of N- and O-glycosylation inhibitors. CD46 expression was determined. In order to assess whether glycosylation of CD46 was important for its functions, we also expressed the different N-glycosylated mutants of CD46 (arginine replaced by a glutamine, as previously published and kindly provided by Dr. JP Atkinson), the O-mutant (region rich in O-glycosylation sites deleted), or the corresponding wild-type isoform, in primary human T cells, and compared the level of CD46 expression, proliferation and cytokine production upon CD46 costimulation.
Results: T cell culture with broad-spectrum inhibitors of N- or O-glycosylation demonstrates contrasting roles of N- and O- glycosylation in CD46 downregulation upon CD46 costimulation. Moreover, expression of the different N- and O- glycosylated mutants has differential effects on CD46 downregulation and T cell function, with antagonistic roles on T cell proliferation and IL-10 production.
Conclusions: Our novel data demonstrate that the level of CD46 glycosylation determines its shedding from the surface upon its activation, which is, in turn, important to control T cell activation. These data also highlight once more the importance of glycans in the control of the immune response.

P0563
Characterization of THEMIS as a new member of the TCR signalosome

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Purpose/Objective: Stimulation of the T cell antigen receptor (TCR) induces formation of phosphorylation-dependent signalling networks, ultimately leading to T cell proliferation and differentiation. Composition and dynamics of the TCR signalosome are still incompletely understood. Others and we have recently identified Thymocyte-expressed molecule involved in selection (THEMIS), as a novel regulator in thymocyte positive selection. The exact role of THEMIS in signalling, especially in mature peripheral T cells, has remained poorly characterized and controversial. Here we set out to study the role of THEMIS in proximal TCR signalling in more detail.
Materials and methods: Work on human T cells was carried out in the Jurkat leukemia T-cell line and in primary human CD4 T cells isolated from peripheral blood. Lentiviral vectors were used for overexpression and knockdown of THEMIS. Tyr-phosphorylation sites were identified on in-vitro phosphorylated recombinant THEMIS by mass spectrometry. THEMIS synapse recruitment was studied by live-cell imaging of GFP-reporters in MHC class II restricted 5C.C7 TCR transgenic mouse T cells.
Results: In the present study we show that THEMIS is a new member of the TCR-proximal signalosome. THEMIS acts as a positive regulator of TCR-induced IL-2 gene expression, via modulation of the MAP kinase ERK and the transcription factor NFAT. TCR ligation leads to rapid Tyr-phosphorylation and recruitment of THEMIS to the immunological synapse via the transmembrane adapter Linker for activation of T cells (LAT). We demonstrate that Lck is the upstream kinase for THEMIS and map potential Tyr-phosphorylation sites. LAT recruitment is essential for THEMIS phosphorylation and is mediated via the adapter molecule growth factor receptor-bound protein 2 (GRB2). GRB2 binds constitutively to a highly conserved proline-rich region (PRR1) at the C-terminus of THEMIS. We can show that GRB2 association to PRR1 is indispensible for THEMIS Tyr-phosphorylation and function *in vitro* and importantly for thymocyte development *in vivo*.
Conclusions: Taken together, our study firmly establishes THEMIS as a regulator of proximal TCR signalling and further shows that correct positioning of THEMIS in the LAT-signalosome via GRB2 is crucial for thymocyte development.

P0564
Characterization of Trojan: a novel leukocyte protein

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Purpose/Objective: Aiming to identify molecules involved in T cell development, we have cloned and characterized a novel avian cell surface protein called 'Trojan' from embryonic thymocyte cDNA library. From our previous results we expect Trojan to have anti-apoptotic and/or proliferative role for lymphocytes. Our current objectives are to investigate its function, its signalling mechanism and characterize the novel protein family it belongs to.
Materials and methods: Trojan characterization. Perform sequence analysis, investigate Trojan expression by RT-PCR and flowcytometry.

Functional analysis. Cells from a T cell line are exposed to different apoptosis inducers (UV light, 5-FU, anisomycin, etc..) and any changes in Trojan expression are measured by flow cytometry. Levels of apoptosis are assessed by Annexin V and 7AAD, while cellular proliferation by CFSE dye loading.

Signalling analysis. Cells are loaded with the tandem Fluo-4 and Fura Red calcium indicators and stimulated with polystyrene beads coated with Trojan-specific antibodies. Elevation in intracellular calcium is measured by flow cytometry.

Protein family characterization. Bioinformatics sequence analyses of the family members are carried out.
Results: Trojan is a transmembrane protein, predicted to have a CCP domain, two Fibronectin type III domains and a short cytoplasmic tail with two possible serine phosporylation sites. Trojan is expressed specifically in lymphoid tissues, while on thymocytes its expression pattern is similar to that of IL-7Rα and the anti-apoptotic Bcl-2.

Upon apoptosis induction in cultured cells, Trojan expression generally rises on the surface of surviving cells and gradually decreases towards its normal levels as cells proliferate.

Stimulation through Trojan has yielded controversial results. We are aiming to mimic cell-cell and ligand-receptor interactions in order to conclude whether Trojan transmits intracellular signalling.

Trojan gene is found in the publicly available databases, where the adjacent two genes encode for hypothetical Trojan-like proteins. One family member is predicted as a receptor type protein tyrosine phosphatase while the other as a membrane protein having pairs of CCP and FN3 domains.

Conclusions: In the presented study we characterize Trojan: a novel cell surface molecule. Trojan has a similar expression during T cell development as IL-7Rα and Bcl-2: two molecules known to be important for thymocyte survival and proliferation. Such similar expression, supported by our current functional analyses, makes Trojan an attractive candidate of having an anti-apoptotic or proliferative role.

P0565

Cord blood leptin levels of healthy neonates are associated with IFN-g production by cord blood T-cells

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Purpose/Objective: Leptin is a hormone synthesized by adipocytes and other tissues, including the placenta, and it regulates food intake and energy expenditure, reproductive and immune functions. The pro-Th1 immunomodulatory effects of leptin have been linked to enhanced susceptibility to autoimmune or inflammatory diseases, mainly in experimental models. Our goal in this study is to investigate the role of leptin in neonatal immunity.

Materials and methods: We measured serum leptin and cytokine (IFN-g, TNF-a, IL-2, IL-4, IL-10, IL-12) levels in cord blood (cb) of 510 healthy neonates, 14 small for gestational age (SGA), 312 appropriately grown for gestational age (AGA) and 184 large for gestational age (LGA). To investigate whether leptin can independently influence cytokine gene expression by cb T-cells and monocytes (Mc), we cultured cb T-cells or Mc, isolated from randomly selected AGA neonates or adult peripheral blood, with leptin.

Results: Median serum leptin concentration in the whole sample was 11 ng/ml. In 11.2% neonates (1 SGA, 32 AGA, 24 LGA), leptin levels were >90th percentile (median 39 ng/ml). In 33.3% of those (3.72% of total sample) with the highest leptin levels (median 46 ng/ml), significantly elevated levels of serum IFN-g were also found (mean 27.11 pg/ml, range 17.5–38.5 pg/ml). In neonates with leptin levels ~50th percentile (median 12 ng/ml) or <10th percentile (median 1 ng/ml), serum IFN-g levels were negligible. All other cytokines measured, were < the assays' detection limits. Leptin can influence cytokine gene expression by cb T-cells and monocytes. Addition of leptin in cb-T-cells and Mc cultures resulted in upregulation of IL-2, IFN-g and IL-4 gene expression in cb and adult T-cells and IL-10 expression mainly in cb-Mc. Significantly higher expression of IFN-g occurred in female cb-T-cells cultured with leptin, compared with male cb-T-cells.

The figure above represents a proposed model of leptin regulation of cytokine production by cord blood T-cells and monocytes.

Conclusions: In conclusion, the concurrent presence of high concentrations in both leptin and IFN-g in cb of healthy infants, and leptin's ability to directly upregulate cytokine gene expression in cb T and Mc cells, indicate that abnormally high leptin levels can independently influence the immune system of healthy newborns, and may mediate gender differences in the development of a Th1 polarized immune response.

P0566

Cytohesins regulate static adhesion, MAP kinase signaling, and T cell receptor internalization in T cells through a GEF-dependent manner

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Purpose/Objective: Members of the cytohesin protein family of guanine nucleotide exchange factors for ARF-GTPases were found to play important roles in immune cell biology, since these proteins are involved in LFA-1-mediated cell adhesion and migration, as well as in MAP kinase signaling during T cell activation. However the exact functions of the individual cytohesins and their precise localizations in the various T cell signaling pathways remain unclear.

Materials and methods: The role of cytohesins during T cell activation was addressed by assessing their contributions to e.g. static or dynamic adhesion, the regulation of TCR internalization, IL-2 production and analysis of MAP kinase signaling.To this end, we employed two recently identified small molecular cytohesin inhibitory compounds (Secin16, Secin144), the specificity of which was proven via surface plasmon resonance affinity measurements and fluorescence-based nucleotide exchange assays.

Results: Analysis of cytohesin activity on the TCR internalization revealed a specific and novel role of cytohesin 3. The overexpression of cytohesin 3, but not of cytohesin-1 or -4, reduced the ligand-dependent and ndependent TCR internalization in a GEF-dependent manner. Furthermore, using the chemical cytohesin inhibitors Secin 16 and Secin 144 for analyses of cell adhesion, MAP kinase signaling, IL-2 production, we observed differential effects. Although Secin16 and Secin 144 both bind to the Sec7 domain of cytohesin 1, only Secin 16 inhibited the nucleotide exchange of the cytohesin Sec7 domain towards ARF 1 *in vitro*. Interestingly, both compounds impaired IL-2 production in stimulated T cells, as well as their adhesion to ICAM-1.On the other hand, we found that the dynamic adhesion of T cell in laminar flow conditions was only inhibited by Secin 144 and was thus GEF-independent, whereas the phosphorylation of ERK 1/2was only impaired by Secin16 and thus GEF dependent.

Conclusions: Therefore we conclude that cytohesin-mediated signaling mechanisms are important for T cell functions, e.g. adhesion, IL-2 production, MAP kinase signaling and T cell receptor internalization, which are GEF-dependent or independent, respectively.

P0568

Differential polarisation of Csk between naïve and antigen-experienced CD8$^+$ T cells

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Purpose/Objective: In CD8$^+$ T cells, engagement of the T cell receptor (TCR) with agonist peptide:MHC molecules causes dynamic redistribution to the immunological synapse of surface molecules including the CD8 co-receptor. CD8 associates with the Src-family kinase (SFK) Lck, which in turn initiates the rapid tyrosine phosphorylation events that drive cellular activation. Compared to naïve T cells, antigen (Ag)-experienced CD8$^+$ T cells make shorter contacts with antigen presenting cells, are less dependent on costimulation, and are triggered by lower concentrations of antigen, yet the molecular basis of this more rapid response of Ag-experienced T cells is not fully understood. Here we show differences in co-localisation of the SFK and their negative regulator, Csk, between naïve and Ag-experienced CD8$^+$ T cells.

Materials and methods: Ag-experienced CD8$^+$ T cells were generated by incubation of naïve F5 TCR Tg CD8$^+$ T cells in 10nM cognate

peptide, NP68, for 3 days and then rested for 4 days in IL-2$^+$ IL-7. Naive and Ag-experienced F5 CD8$^+$ T cells were stimulated with TCR$^+$CD8 cross-linking for 5mins, then fixed in 4% PFA and permeabilised in Triton X-100 for double staining of pTyr, Lck, LckpTyr^{505} and Fyn with Csk. Cells were imaged by 4-colour confocal microscopy and analysed by Volocity and ImageJ software.
Results: In naïve CD8$^+$ T cells there was pronounced co-localisation of the SFK and Csk at the immunological synapse, while in Ag-experienced cells, although more Lck molecules redistrbuted to the proximal pole of the cells, Csk displayed a bipolar distribution. Interestingly, the SFK Fyn, shared a similar bipolar distribution as Csk in Ag-experienced CD8$^+$ T cells. Furthermore, in Ag-experienced but not naïve CD8$^+$ T cells, a proportion of Csk was sequestered within a cytosolic structure.
Conclusions: The data show that there is differential redistribution of a key negative regulator away from the site of TCR engagement in Ag-experienced compared to naïve CD8$^+$ T cells, which might be associated to the more rapid responses of memory cells upon re-exposure to antigen.

P0569

Differentially expressed genes in MiHA-specific CD8$^+$ T cells upon co-inhibitory receptor-mediated T cell dysfunction after allogeneic stem cell transplantation

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Purpose/Objective: Allogeneic stem cell transplantation (alloSCT) can cure hematological malignancies by inducing alloreactive T cell responses targeting minor histocompatibility antigens (MiHA) expressed on malignant cells. Despite induction of robust MiHA-specific T cell responses and long-term persistence of alloreactive memory T cells specific for the tumor, often these T cells fail to respond efficiently to tumor relapse. Previously, we demonstrated the involvement of the coinhibitory receptors programmed death-1 (PD-1) and B and T lymphocyte attenuator (BTLA) in suppressing MiHA-specific CD8$^+$ T cell immunity. Signalling via these and other receptors have rendered the alloreactive T cells dysfunctional. The aim of this study is to identify which key intracellular mediators are involved in the exhaustion of MiHA-specific T cells.
Materials and methods: To characterize diferences between functional versus impaired MiHA-specific memory CD8$^+$ T cells, we have isolated cells by flow cytometry assisted cell sorting. These samples were either from 10 patients in remission (functional) or from 10 patients which developed relapse, despite initial immune responses (impaired). Next, we compared gene expression profiles between these groups on a dedicated qPCR platform. Genes investigated were selected after an explorative microarray analysis and literature search.
Results: By comparing gene expression profiles of MiHA-specific T cells from patients in remission versus patients whom experienced relapse, we found interesting differences. As expected, co-inhibitory molecules were differentially expressed between these two groups. Furthermore, we found differences in effector molecules such as Granzyme B and perforin. Interestingly, we found a higher expression of the transcription factor FOXO3 in MiHA-specific memory CD8$^+$ T cells of relapsed patients. FOXO3 is a pro-apoptotic transcription factor, and has been implicated in T cell memory formation. These factors provide a possible link to co-inhibitory receptor-mediated T cell impairment, and therefore the role of FOXO3 in this process will be investigated.
Conclusions: We have identified interesting differentially expressed genes, by comparing gene expression profiles from MiHA-specific T cells from relapsed versus patients in remission. These include co-inhibitory molecules, T cell effector proteins, and interestingly, the transcription factor FOXO3. Therefore, the role of FOXO3 in immune responses after alloSCT needs to be investigated, and may provide a rationale to prevent of reverse T cell functional impairment of MiHA-specific memory CD8$^+$ T cells.

P0570

Distinct signalling patterns and functional responses to CD2 and CD28 co-stimulation of human primary T cells

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Purpose/Objective: T-cell activation depends on stimulation of the T-cell receptor (TCR) via interactions with peptide/ MHC molecules on antigen-presenting cells (APCs) together with additional signals from co-receptors. Co-receptor ligation modulates the intracellular signalling pathways that regulate T-cell fate and lack of co-stimulation results in non-responsiveness or T-cell anergy. Studies have demonstrated that CD28 is the principal co-stimulatory receptor for T-cell activation, but multiple molecules on the T cell have been described to deliver co-stimulatory signals. CD2 functions as an adhesion molecule, but cross-linking of CD2 also serves as an efficient co-stimulus in T cells and CD28 and CD2 have been considered largely redundant based on studies with null mutant mice. We are currently investigating the differences in CD2 versus CD28 co-stimulation on downstream signalling events in human primary T cells.
Materials and methods: Using phospho-specific flow cytometry in combination with fluorescent cell barcoding, we are mapping both proximal and distal T-cell signalling events under various co-stimulatory conditions. Peptide array technology enables characterisation of the interactions between the Lck/Fyn SH3-domains and CD2/CD28.
Results: CD2 and CD28 co-stimulation trigger overlapping but distinct signalling pathways in human primary T cells. Phospho-specific flow cytometry analysis revealed that stimulation of CD28 is required for the activation of NF-κB while stimulation through CD2 appears to give stronger signalling responses in more TCR-proximal proteins such as SLP-76. Furthermore, CD2 and CD28 co-stimulation result in different cytokine responses. Both CD2 and CD28 associate with the Src family tyrosine kinases Lck and Fyn via proline-rich sequences contained within their cytoplasmic domains. To investigate the molecular mechanisms that give rise to the distinct signalling responses observed after CD2 and CD28 co-stimulation, we are dissecting the protein interactions between the co-receptors and Lck/Fyn.
Conclusions: Our study describes distinct differences, both qualitatively and quantitatively, in the co-stimulatory capacities of CD2 and CD28.

P0572

Identification of enzymes that dephosphorylate Ser39 of PTP-PEST in CD3/CD28-mediated phosphorylation

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Purpose/Objective: Protein tyrosine phosphatase-PEST (PTP-PEST), encoded by the *PTPN12* gene, is a protein tyrosine phosphatase associated with T cell signaling. Previous studies have associated PTP-PEST with TCR regulation, however, the specific role has not been fully elucidated. PTP-PEST has two phosphorylation sites; Ser39 and Ser435, the former of which is the major site that negatively regulates

the PTP activity. So far we have demonstrated that Ser39 phosphorylation is induced in CD3/CD28 costimulated Jurkat T cells, predominantly through the PKC-θ. However, molecules involved in the dephosphorylation observed after 10 min stimulation, have not yet been established. We identified PP1α as a PTP-PEST binding protein that associates with the dephosphorylation of Ser39, and postulate the possible participation of PP1-isoenzymes to be involved in the dephosphorylation of CD3/CD28-costimulated Jurkat T cells. In this study, we aim to determine the Ser39 dephosphorylating enzymes in CD3/CD28-costimulated Jurkat T cells, focusing on PP1 isoenzymes.
Materials and methods: Jurkat T cells expressing exogenous PTP-PEST pretreated with and without PP1-specific inhibitors were stimulated by anti-CD3/anti-CD28 mAbs, and the phosphorylation of PTP-PEST at Ser39 was evaluated by Western blotting with phosphor-Ser39-specific antibody. HEK 293 cells expressing exogenous PTP-PEST and PP1 isoenzymes were treated with TPA and Ser39 phosphorylation was determined by Western blotting.
Results: Ser39 was phosphorylated by CD3/CD28-costimulation, and dephosphorylation observed after 10 min stimulation in Jurkat T cells. Cells treated with PP1-specific inhibitors had pronounced CD3/CD28-mediated Ser39 phosphorylation, compared with untreated cells. TPA-mediated Ser39 phosphorylation was diminished by PP1α, PP1β and PP1γ1, suggesting that PP1 isoenzymes contributed Ser39 dephosphorylation in Jurkat T cells.
Conclusions: Ser39 of PTP-PEST was phosphorylated by CD3/CD28-mediated signaling, and subsequently dephosphorylated. Here we identified PP1 as major dephosphorylating enzymes of Ser39, however, the isoenzyme specificity is presently under investigation.

P0573
Importance of growth factor receptor-bound protein 2 (Grb-2) binding to CD28 in mediating NF-kB activation in T cells

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Purpose/Objective: The full activation of T-cells is achieved via concomitant engagement of CD3/TCR and CD28 molecules on T-cells (1, 2). While the role of the co-receptor CD28 in T-cells is well established, the nature of their interdependence in the NF-kB activation is poorly understood (2). Whether TcR/CD3 and CD28 operate as separate entities, or need to cooperate for NF-kB activation is not well established.
Materials and methods: CD28 deficient (i.e. *Cd28*$^{-/-}$) primary T-cells and Jurkat T-cells were used to monitor NF-kB activation using dual luciferase assay and electro-mobility shift assay supplemented with flow cytometry and western data. Role of cytoplasmic domain of CD28 was investigated using site specific knock-in mouse model or CD28 deficient cells reconstituted with mutant (N193Q) CD28 that abrogated Grb2 binding. Further, Grb2 expression was specifically knocked down using siRNA.
Results: We previously showed that CD28 binds to the growth factor receptor-bound protein 2 (Grb-2) via the YMNM motif (3,4). Here, we show, using *Cd28*$^{-/-}$ primary T-cells and Jurkat T-cells, that substantial NF-kB activation can be achieved by CD28 ligation alone in the absence of antigen receptor ligation. Further, using a site specific mutant in the CD28 domain at YMN-QM that disrupts Grb-2 binding to cytoplasmic domain, we showed that this interaction is critically needed for NF-kB activation. Similarly, siRNA knockdown of Grb-2 expression prevented NF-kB activation as shown by luciferase and EMSA assays. The additional role of CD28-Grb-2 interaction with downstream adapter molecules for NF-kB activation will be discussed.
Conclusions: Co-stimulatory molecule CD28 forms an essential component of NF-kB activation during T cell activation. CD28 mediated NF-kB activity is dependent upon its binding to Grb2 and does not essentially requires T-cell receptor co-engagement.

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P0574
Inhibition of peptidyl prolyl cis-trans isomerases decreases CrkII association with its functional binding partner in leukemic T cell

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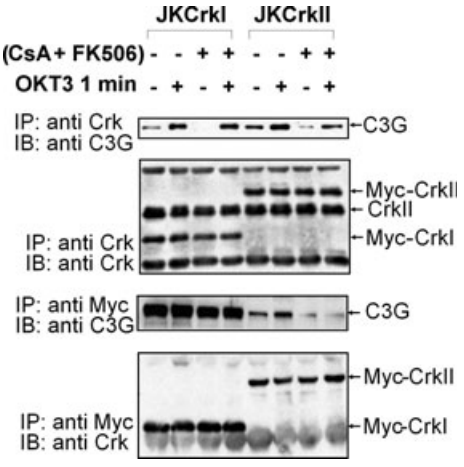
Purpose/Objective: Cell growth and differentiation are strictly controlled processes mediated by effector molecules, which are regulated by posttranslational chemical modifications. Adaptor proteins are critical players in these processes, thanks to their ability to simultaneously interact with two or more effector molecules and orchestrate the assembly of signaling complexes downstream of activated surface receptors. Among these adaptor proteins, CrkII is ubiquitously expressed, involved in multiple receptor-linked signaling pathways, and involved also in signal transduction downstream of activated T cell antigen receptors. Peptidyl prolyl cis-trans isomereses (PPIases) are widely distributed proteins and has recently emerged as important molecular timers in the dynamic regulation of biological processes. We aimed to determine whether CrkII can serve as an *in vivo* substrate for PPIases and whether inhibitors of PPIases can alter CrkII-dependent T cell activation responses.
Materials and methods: CrkII-based binding studies using PPIases-inhibitors treated or untreated T cells, a FRET-based assay, immunofluorescence assay by confocal microscopy and studies of the effect of the inhibitors on CrkII-dependent T cell functions, including cell adhesion and migration.
Results: Treatment of Jurkat T cells with the inhibitors decreased the ability of CrkII-SH3N domain to associate with binding partners. In parallel, this treatment altered the proportions of *cis* versus *trans* forms of CrkII, as indicated by FRET studies. Finally, CrkII overexpression-induced increase of Jurkat T cell adherence and migration was significantly inhibited by pretreatment with PPIases-inhibitors.



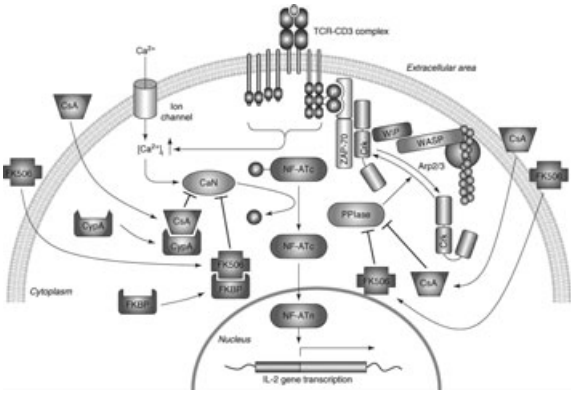


Figure 1. Intervention of distinct signaling pathways in activated T lymphocytes by Cyclosporine A (CsA) and FK506.

Conclusions: Our results suggest that CrkII can serve as an *in vivo* substrate for PPIases in Jurkat T cells. Inhibition of CrkII association with binding proteins by PPIases-inhibitors may represent one additional mechanism of PPIases-mediated inhibition of T cell activation, which potentially synergizes with the negative effect of PPIases-inhibitors on the calcineurin-NF-AT signaling pathway.

P0575

Integration of activating and inhibitory immune-receptor signaling by regulated Vav1 phosphorylation

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Purpose/Objective: Natural Killer (NK) cells are important immune effector cells whose activation is carefully regulated by the interplay of activating and inhibitory receptors signals. Triggering of activating receptors leads to activation of Src family kinases and Vav1 phosphorylation, whereas inhibitory receptors dephosphorylate Vav1 via the phosphatase SHP-1.
Materials and methods: Here we use mathematical modeling confirmed by experimental data to gain insight into this integration of positive and negative signals on a molecular level.
Results: Based on published signaling events a simplified model of the receptor proximal events of NK cell activation was generated. Six putative signaling modules that could influence the activation can be included into the model. Our experiments showed that increased triggering of activating receptors lead to a rapid switch-like increase in Vav1 phosphorylation. Similarly, titrating the engagement of inhibitory receptors resulted in switch-like dephosphorylation of Vav1. NK cell cytotoxic activity correlates with phosphorylation of Vav1. Comparing experimental results with predictions derived from the family of mathematical models shows that an association of Src-family kinases with activating receptors is essential to create such a physiologic response. Interestingly, other concepts of immune receptor signaling such as phosphatase segregation and kinase auto-phosphorylation were not necessary in our mathematical model.
Conclusions: Our data support a central role for Vav1 in the decision making process of NK cells and provide a novel insight into the molecular details of the integration of positive and negative signals during lymphocyte activation.

P0576

Interplay between receptors of the adaptive and innate immune systems in human B-cells

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Purpose/Objective: Development of B-cells is regulated by the antigen binding receptors (BCR) and a bunch of other receptors positively or negatively influencing the cells' fate. The innate receptor, TLR9 is located on intracellular membranes (endosomes) and may use BCR as a shuttle to deliver its ligand, hypomethylated CpG rich DNA sequences. The B-cell activating factor, BAFF mediates survival signals for B-cells thus support antigen driven development. The communication between signaling pathways mediated by BCR, TLR9 and BAFF receptor (BAFFR) is not clarified yet. Efficient co-signaling may lower the activation threshold of B cells, leading to the development of B-cells with low affinity BCR that are potentially autoreactive. Our aim was to identify crucial molecules responsible for the cross-talk between signaling pathways thus playing role in breaking the tolerance.
Materials and methods: For the parallel determination of the relative levels of phosphorylation of mitogen activated protein kinases (MAPKs) and other serine/threonine kinases human phospho-MAPK array kit was used. We confirmed and further studied the results by Western blot technique and phospho-flow analysis. Cell proliferation was measured by CFSE staining of B cells; the results were evaluated by flow cytometry. Cytokine secretion was tested by Flow-Cytomix kit.
Results: We have found that TLR9 ligation induces p38, JNK and MSK2 activation in synergy with the BCR induced signals. Upstream of MAPK, the MAPKKK7/Tak1 is synergistically activated via BCR and TLR9 but not by BAFF. BCR-induced Erk1 phosphorylation is slightly enhanced both by TLR9 and BAFFR mediated signals, while TLR9 and BAFFR do not cooperate at this level. Downstream on the pathway, the BCR-induced CREB phosphorylation is enhanced both by CpG DNA and by BAFF, while IκB phosphorylation is synergistically induced by TLR9 and BAFF or BCR, respectively. Functional assays have shown that BCR and TLR9 mediated collaborative signals significantly stimulate B-cell proliferation that can be diminished by Tak1 inhibitor. Furthermore, BCR and TLR9 mediated signals synergistically operate in inducing secretion of IL-6 and IL-10; and this effect is also blocked by specific inhibitor of Tak1.
Conclusions: Our results suggest that Tak1 playscentral role in the interplay between BCR and TLR9 mediated signalling and in regulation ofB-cells' function.

P0577

Jak3 enables T cell migration towards chemokines via Cofilin-1 and RhoA

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Purpose/Objective: Jak3 is involved in the CXCR4- and CCR7-dependent T cell migration. As cytoskeletal dynamics are essential to allow migration, we investigated the impact of Jak3 deficiency in CXCR4 and CCR7 mediated cytoskeleton reorganization involved in this process.
Materials and methods: Lymph node T cells from C57BL/6 mice or human PBMCs were treated or not with the Jak3 specific inhibitor WHI-P131 or Pertussis toxin (PTX). In addition, parallel experiments

were performed using splenic T cells of Jak3$^{-/-}$ or Jak3$^{+/+}$ mice. Cells were stimulated with the chemokine CXCL12 (human cells) or CCL21 (murine cells) at different time points. Cell shape changes and polymerized actin in response to chemokines were analyzed by time-lapse microscopy or flow cytometry, respectively. Western blot analysis was performed to analyze cofilin phosphorylation kinetics. RhoA activation was analyzed by fluorescent microscopy staining of RhoA-GTP.

Results: Time-lapse analysis showed that WHI-P131-treated cells display impaired motility and deficient uropod formation towards chemokines. Secondly, actin polymerization was impaired in the absence of Jak3 activation in response to CXCL12 and CCL21, in both pharmacologically inhibited and Jak3$^{-/-}$ T cells. This correlates with the cofilin phosphorylation kinetics observed in WHI-P131-treated and non-treated cells. Non-treated cells have a peak of activation (dephosphorylation) at 30 s post stimulation and returns to the basal level at 300 s, while in WHI-P131-treated-cells cofilin activation continues increasing after 30 s up to 300 s. In addition, confocal microscopy revealed that RhoA-GTP was significantly diminished in WHI-P131-treated-cells.

Conclusions: We have demonstrated the involvement of Jak3 in the actin dynamics in response to chemokines. Time-lapse and confocal microscopy analysis suggests a link between Jak3 and RhoA for uropod formation. Moreover, F-actin reduction may be a consequence of active cofilin levels post stimulation. In summary, our results provide strong evidence that T cells require Jak3 to achieve appropriate actin dynamics, cofilin kinetics and RhoA activation and translocation in response to CXCL12 and CCL21. Work supported by PAPIIT, DGAPA, UNAM.

P0578
Modulating p56Lck signalling in T-cells by a chimeric peptide derived from two independent Herpesvirus saimiri Tip motifs

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Purpose/Objective: To study the effect of the Lck binding domain CSKH from Herpesvirus samiri Tip protein in human and Aotus T-cell signalling and activation. To test the proper delivery of this motif to lipid rafts by using an hydrophobic sequence motif from the Tip protein.

Materials and methods: Peptides, including the hydrophobic Tip sequence (hTip), the Tip Lck-binding motifs (CSKH) and the chimera (hTip-CSKH), were synthesised by solid phase synthesis. Human and Aotus PBMC were cultured for 1 h in the absence or presence of different peptide concentrations, and stimulated or not with PHA-P or PMA. Proliferation was assayed by [methyl-3H]-thymidine incorporation. Immunodetection was performed with anti-Lck, anti-phosphotyrosine, anti-p-ERK1/2, anti-ERK1/2, anti-Fyn, anti-β-Actin and anti-Flotillin-2. Rafts were isolated by sucrose density gradients. Surface marker expression was evaluated with anti-CD3; anti-CD25 and anti-TCRα/β antibodies.

Results: Here we have shown thathTip-CSKH can induce an increased proliferation rate in either unstimulated or stimulated conditions (PHA-P). This effect was dose-dependent and was not observed with control peptides.hTip-CSKH modifies the Lck electrophoretic pattern and phosphorylation state, simulating the changes induced by polyclonal T-cell receptor stimulation. Nevertheless, phosphorylation kinetics was increased with the chimeric peptide. Furthermore, treatment with this peptide complements the partial signal induced by phorbol esters, confirming its specific effect on T-cell receptor signalling. hTip-CSKH was present high density lipid rafts, as it would be expected for an Lck activating molecule. By treating cells with this short sequence we did not observe any downmodulation of cell surface marker expression, as occurs with transfection of the whole Tip molecule. Also, it was shown that Tip effect is specific for Lck activation, with no activation of Fyn.

Conclusions: This approach let us to conclude that Tip's CSKH motif can modulate specifically T-cell receptor pathways by targeting Lck. This could be the basis for designing specific T-cell immunomodulating compounds directed to target molecules present in lipid rafts. This approach would be also useful for some diseases in which rafts are involved during signaling.

P0579
OX40L engagement induces a reverse signalling in human B cells

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Purpose/Objective: The tumour necrosis factor superfamily member 4 gene, which encodes for the co-stimulatory molecule OX40 ligand, has been previously identified in our lab as a susceptibility gene in SLE. It is well known that the engagement of OX40 through OX40L provides a co-stimulatory signal promoting division, survival and clonal expansion of effector and memory T-cells; in contrast, very little is known about OX40L intracellular signalling and the role that this could play in autoimmunity. In this study we want to investigate the OX40L reverse signalling pathway, in particular its effects in human B-cells.

Materials and methods: A soluble OX40 fusion protein (OX40-FP) was produced by cloning the extracellular domain of OX40 into a vector in which a C-terminal double-tag was integrated by oligonucleotide cloning. Binding of OX40-FP was assessed by competition assay using an antibody to OX40L. OX40-FP was used to trigger OX40L in human B cells stimulated with F(ab')2 anti-IgM and CD40L and to perform a tandem affinity purification of potential OX40L binding partners.

Results: EBV transformed B cells (EBV-B cells) expressing OX40L were used to test whether the OX40-FP binds OX40L. The specificity of the binding was shown by a decrease in OX40-FP binding on EBV B cells pre-treated with increasing amount of an antibody to OX40L. We found that B cells treated with OX40-FP showed less proliferation ($P < 0.05$). Furthermore we observed a higher production of IgG in B cells activated through OX40L engagement compared to control cells. We could not observe any difference in Ca^{++} mobilization in cells pre-treated with OX40-FP. Potential OX40L binding partners were isolated by stimulating EBV-B cells with OX40-FP and immune-precipitating the OX40-OX40L complex using the two tags of the OX40-FP. Performing an SDS-page followed by silver staining we could show the binding of several different proteins to our complex.

Conclusions: Using a recombinant OX40 protein as a molecular tool we have been able to show how engagement of OX40L initiates reverse signalling in human B-cells leading to less proliferation and increase of IgG production. Moreover, the design of our OX40-FP allowed us to start to investigate the molecular basis of OX40L reverse signalling. Several proteins have been already purified and are being characterized by liquid chromatography-tandem mass spectrometry.

P0580
Physiopathology of TCR/CD3: genomic induction in CD3gamma deficient cells

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Purpose/Objective: The final objective of this project is the analysis by means of the technique of microarrays in order to evaluate the geno-

mic induction in transformed T lymphocytes which are CD3gamma deficient ($\gamma^{-/-}$) after stimulating with anti-CD3 antibodies, comparing these results with the ones obtained in normal lymphocytes ($\gamma^{+/+}$).
Materials and methods: Comparative flow cytometry, Western-blot, oligonucleotide microarray.
Results: It was proved that it is possible to stimulate transformed T cells (*Herpesvirus saimiri*) despite of their high basal activation level. We have checked this stimulation analyzing early functional parameters (CD69 and CD25 induction, calcium flux induction) and late ones (phosphorylation pattern). After anti-CD3 stimulation of the cells (both $\gamma^{+/+}$ and $\gamma^{-/-}$), cDNA was hybridized against a chip and afterwards, gene expression estimates were calculated.
Conclusions: There is a different gene expression pattern between CD3gamma deficient and sufficient transformed T cells. There are many genes which are differently induced (upwards or downwards) and this difference is attributable to CD3gamma chain.

P0581
Protein kinase D2 integrates antigen receptor, diacylglycerol and Protein Kinase C mediated signals to fine tune cytokine production in T cells

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Purpose/Objective: Protein kinase Ds are a conserved family of serine/threonine kinases. PKD2 is the predominant PKD isoform expressed in lymphocytes, and it is selectively phosphorylated and activated in response to T cell receptor (TCR)-induced generation of diacylglycerol and stimulation of PKC. Our aim is to explore the role of PKD2 in T cells.
Materials and methods: To analyse the role of PKD2 in T cells we have generated PKD2 null mice and mice deficient in PKD2 enzymatic activity ('knockin' mutation of phosphorylation sites essential for PKD2 catalytic activity), and we have backcrossed them in to TCR-transgenic mice (P14 and OTI).
Results: Mice that lack PKD2 or have wild type PKD2 alleles substituted with PKD2 mutant alleles that cannot be phosphorylated by PKC produce peripheral T cells that have the transcriptional profile of normal naïve T cells. However, these cells have defects in antigen receptor induced proliferation and they are unable to correctly reprogram their transcriptome in response to TCR engagement. The role of PKD2 in mediating the TCR induced transcriptional program is selective but includes the control of key cytokines and chemokines, like interleukin 2 and interferon gamma, known to be essential for T cell effector function. PKD2 thus has a restricted but vital role in antigen receptor signal transduction. PKD2 is required for the effector function of cytotoxic T cells. The present study presents a quantitative analysis of PKD2 deficient cytotoxic T cell phosphoproteome using high resolution quantitative mass spectrometry as a screening tool for putative PKD2 substrates.
Conclusions: PKD2 activity is required for maximal TCR-induced clonal expansion and differentiation into CD8 effector T cell. PKD2 is involved in diverse processes that control expression of essential pro-inflammatory cytokines. PKD2 has a global impact in the T cell phosphoproteome, revealing both direct and indirect pathways controlled by PKD2 catalytic activity.

P0582
Regulation of ERM (Ezrin/Radixin/Moesin)-activity during LFA-1-mediated T cell migration

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Purpose/Objective: T cell migration requires adhesion at the cell-front and de-adhesion at the back, controlled by interaction of active LFA-1 with its ligand ICAM-1 and dissociation upon LFA-1 de-activation. Rearrangement of the cytoskeleton regulates cell migration intracellularly. ERM (Ezrin/Radixin/Moesin)-proteins act as linker between the membrane and the cytoskeleton. We were interested in the role of ERM-proteins in mediating de-adhesion of migrating T cells by signal-transduction from LFA-1 to the cytoskeleton.
Materials and methods: We investigated the localization of ERM-proteins in primary murine T cells migrating on ICAM-1 by confocal microscopy and their interaction with LFA-1 by immunoprecipitation. We compared expression of phosphorylated ERM-proteins in migrated T cells expressing wildtype or constitutively active LFA-1. We screened for phosphatases regulating ERM activity. In order to investigate the role of identified phosphatases during T cell migration we chemically interfered with their activity in naïve T cells and analyzed LFA-1 mediated T cell migration by live-cell imaging *in vitro*, and homing experiments to lymph node and spleen *in vivo*.
Results: We found ERM-proteins being co-localized with LFA-1 at the site of de-adhesion, the back end of migrating cells, but not directly interacting with LFA-1. The equilibrium between active and inactive ERM-proteins was shifted towards the active, phosphorylated form in T cells expressing constitutively high affinity LFA-1 and showing a defective de-adhesion. This indicates that increased ERM-phosphorylation might correlate with defective T cell migration. We identified the protein phosphatase PP1 to interact with LFA-1 in T cells and PP1/PP2A to specifically dephosphorylate ERM-proteins. Blocking PP1/PP2A-activity in T cells impaired T cell migration *in vitro* and *in vivo*. Namely, T cells did not actively migrate across endothelial cells *in vitro*, and were not able to enter the lymph node T cell zone or the white pulp of the spleen *in vivo*.
Conclusions: We identified PP1 as binding partner of LFA-1 in T cells and demonstrate that PP1/PP2A specifically dephosphorylate ERM-proteins. Our data provide evidence for the importance of ERM-activity-regulation by PP1/PP2A, downstream of LFA-1, during T cell migration.

P0583
Regulation of T cell activation mediated by modification and localization of the Src family kinase Lck

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Purpose/Objective: p56Lck, the T cell specific non-receptor protein tyrosine kinase, plays a key role in signaling from the T cell antigen receptor (TCR). According to common 'textbook' knowledge, Lck is supposed to undergo structural changes from a closed inactive to an open active conformation upon TCR engagement. However, previously we could show by biochemical assays and live-FRET imaging that regulation of Lck upon TCR stimulation is rather mediated by other mechanisms, presumably reorganization of localization or domain displacement of intramolecular interactions by ligands than acute structural changes of the molecule.

Materials and methods: A variety of mutated and truncated Lck molecules were generated and C-terminally tagged with a monomeric enhanced green fluorescence protein (mEGFP). These constructs were retrovirally transduced into Lck-deficient JCaM1.6 Jurkat T cells and used for biochemical and microscopy experiments.
Results: We investigated the determinants mediating binding of Lck to the inner leaflet of the plasma membrane, and also determined the ratio between cytoplasmatic and membrane-bound Lck molecules in stimulated and non-stimulated T cells. By using a mEGFP-tagged Lck biosensor and a non-invasive single molecule imaging approach we were able to determine the exact lifetime of Lck in the plasma membrane. Moreover, we found by live-FRET imaging and single molecule analysis Lck molecules forming dimers and higher order structures. Biochemical methods showed that the N-terminal membrane anchor is mediating this intermolecular interaction of Lck.
Conclusions: Together, these advanced imaging studies of Lck in the live cell context provide a novel picture of the function and regulation of this key kinase in signaling via TCR.

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P0584

SLAM family mediated cyotoxicity in NK cells depends on PLCγ1 and PLCγ2

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Purpose/Objective: The mechanism of signal transduction by receptors containing Immunoreceptor Tyrosine-based Switch Motifs in the CD2/SLAM family is of interest particularly because of the disease association with X-linked proliferative disease and autoimmunity, and potential exploitation in treating cancer. Two related intracellular SH2 domain containing adaptor proteins, SAP and EAT-2 bind the tyrosine motifs. Transmission of activating signals through SAP involves simultaneous, non competitive binding to cytoplasmic tails of SLAM family receptors and SH3 domains of signalling proteins. EAT-2 also has an activating role downstream of SLAM family receptors, which is dependent on phosphorylation of a C-terminal tyrosine motif. The purpose of this study was to determine the direct binding partners for the C-terminal tyrosine motif in EAT-2 and test the functional relevance of the biochemical data.
Materials and methods: We have used a biochemical approach involving mass spectrometry and surface plasmon resonance and carried out functional experiments in an NK cell line using shRNA.
Results: We identify interactions between EAT-2 and PLCγ1 and PLCγ2 likely to be important for regulation of signal transduction by SLAM family receptors. Functional data have provided insight into the relative importance of adaptors, EAT-2 and SAP and enzymes, PLCγ1 and PLCγ2 for SLAM family mediated cytotoxicity in a human NK cell line.
Conclusions: SLAM family mediated cyotoxicity in NK cells depends on PLCγ1 and PLCγ2. Correlation between dependence of a SLAM family receptor, CRACC on EAT-2 and PLCγ is consistent with a direct interaction between them.

P0585

T-cells stimulated via LFA-1/ICAM-1 are programmed to become refractory to TGF-ß signalling: Implications for local inflammatory response

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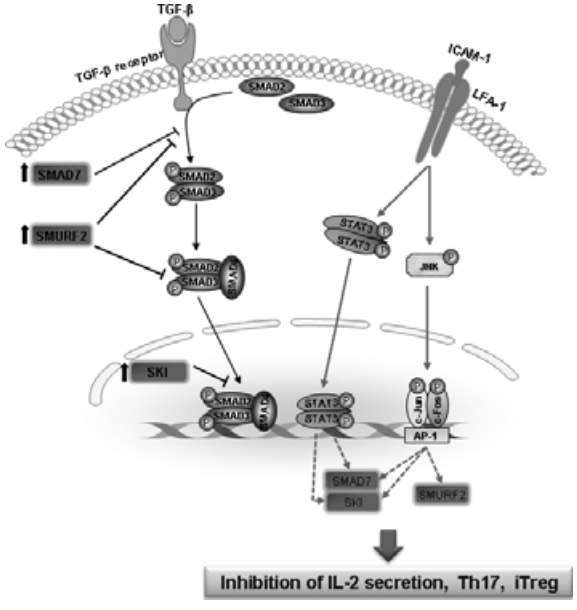
Purpose/Objective: The β_2 integrin LFA-1 plays a key role in lymphocyte motility, but the downstream gene regulation by this adhesion molecule remains largely unknown. The aim of this study was to investigate changes in gene expression caused by the LFA-1 interaction with its ligand ICAM-1, and subsequent functional consequences in T-cells.
Materials and methods: Peripheral blood lymphocyte T-cells from healthy volunteers and the human T-cell line Hut78 were used. Differential gene expression in T-cells was identified by cDNA microarrays combined with gene ontology and Ingenuity Pathway Analysis. Standard molecular, biochemical and microscopic techniques were employed.
Results: The LFA-1/ICAM-1 interaction induced a genetic signature associated with reduced TGF-β responsiveness *via* up-regulation of SKI, SMURF2 and SMAD7 genes and proteins. The expression of these TGF-β inhibitory proteins was dependent on STAT3 and/or JNK activation. Increased expression of SMAD7 and SMURF2 in LFA-1/ICAM-1 cross-linked T-cells resulted in impaired TGF-β-mediated phosphorylation of SMAD2 and suppression of IL-2 secretion. Expression of SKI caused resistance to TGF-β-mediated suppression of IL-2, but SMAD2 phosphorylation was unaffected. Blocking LFA-1 by neutralizing antibody or specific knockdown of TGF-β inhibitory molecules by siRNA substantially restored LFA-1/ICAM-1-mediated alteration in TGF-β signalling. LFA-1/ICAM-1 stimulated T-cells were refractory to TGF-β-mediated induction of FOXP3$^+$ Treg and RORγt$^+$ Th17 differentiation.



Conclusions: This study reveals a complex molecular programme that links gene transcription induced by LFA-1/ICAM-1 interaction to impairment of TGF-β signal transduction in T-cells. Given a crucial role of the immunosuppressive cytokine TGF-β in the induction and maintenance of immunologic tolerance, these new findings form a basis of future translational research for human diseases and for designing of immunotherapies.

P0586

The absence of individual NFAT family members ameliorates experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis

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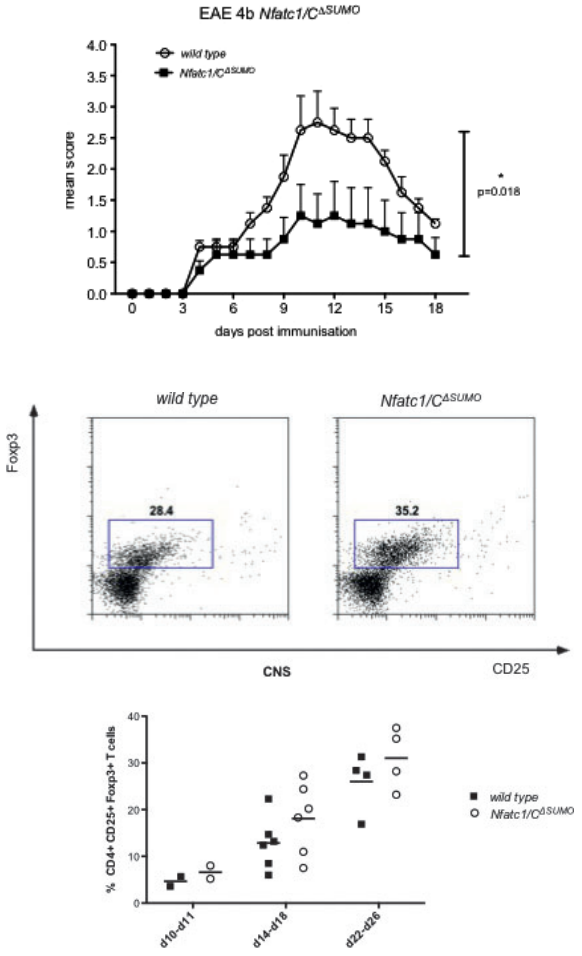
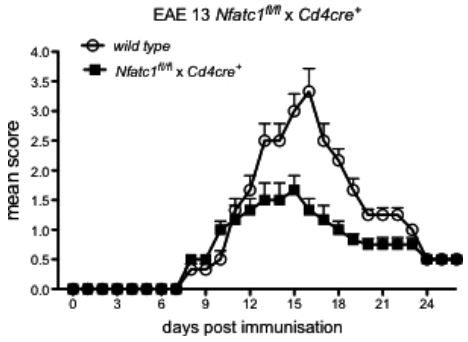
Purpose/Objective: Multiple sclerosis (MS) is an autoimmune disease, where after inflammatory disruption of the blood brain barrier immune cells infiltrate the CNS and cause axonal damage and demyelination within the CNS. T helper cells, in particular Th1 and Th17, are important mediators during this progress and are antagonized by regulatory T cells (Treg). Members of the transcription factor family 'Nuclear Factors of Activated T cells' (NFAT) are induced in response to TCR stimulation and act as pleiotropic regulators of T cell function.

Materials and methods: To investigate the role of single NFAT factors in the pathophysiology of multiple sclerosis we used several NFAT deficient mice in the animal model of this disease (EAE).

Results: So far we could show that not only the deficiency of NFATc1 or NFATc2, but also the absence of the C-terminal (specific) sumoylation of NFATc1 reduces the clinical severity of EAE. Regardless





of a comparable influence on the disease, the reasons are distinct: T cells from *Nfatc1/C*$^{\Delta SUMO}$ mice produce significantly more IL-2 *in vitro* and *in vivo*, especially under Th1 driving conditions, while cultured Th1 and Th17 express less IFNγ and IL-17, respectively. Consistently, the MOG_{35-55}-induced EAE with *Nfatc1/C*$^{\Delta SUMO}$ mice revealed an ameliorated clinical disease severity compared to *wild type* (*wt*) controls with a robust IL-2- driven increase in Tregs and a reduction in effector lymphokine producing T helper cells. In case of the T cell specific loss of NFATc1 (*Nfatc1*$^{fl/fl}$ × *Cd4cre*$^{+}$ mice) the clinical score was diminished due to impaired effector functions of $CD4^+$ T cells lacking all NFATc1 isoforms. This was demonstrated by lower levels of IFNγ and IL-17 producers in the CNS of *Nfatc1*$^{fl/fl}$ × *Cd4cre*$^{+}$ mice compared to *wt* siblings. Unexpectedly, also the *Nfatc2*$^{-/-}$ mice showed a protection against experimental autoimmune encephalomyelitis. The deficiency of NFATc2 results in a striking defect of $CD4^+$ T cells in producing IFNγ, which might be ascribed to the known enhanced immune responses with Th2-like characteristics of *Nfatc2*$^{-/-}$ mice.

Conclusions: In summary, we could show that individual NFAT factors and their isoforms/modifications play a distinct role in the pathogenesis of MS. Therefore, targeting or modulating specific NFAT factors could be a future therapeutic approach to inhibit undesired or boost desirable NFAT functions in the respective human disease context.

P0587

The ITIM receptor CD31 is recruited to the immunological synapse to act as a negative co-receptor of the TCR

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Purpose/Objective: CD31 is a homophilic ITIM receptor expressed by endothelial cells, platelets and leukocytes, that comprises 6 Ig-like extracellular domains. Trans-homophilic engagement of the distal domain drives: 1- Clusterization of CD31 molecules at the cell surface via cis-homophilic interaction of a juxtamembrane extracellular peptide sequence 2- ITIMs phosphorylation that activates of SHP-2. CD31 is cleaved following TCR stimulation. Although the membrane-proximal portion, containing the sequence of clusterization, remains expressed after the cleavage, the trans-homophilic domain is lost. We therefore reasoned that CD31 cleavage occurs at the level of the immunological synapse, where otherwise its trans-homophilic engagement by the interacting antigen-presenting cells would prevent effective activation.

Materials and methods: We studied the localization and integrity of CD31 molecules on Jurkat T cells during immunological synapse formation and assessed the role of CD31 on T cell activation by using $CD31^-$Jurkat $CD4^+$ T cells or a peptide able to trigger the signaling

downstream of a cleaved CD31. Immunological synapses were generated by crosslinking the MHC II of RAJI (Activated B cells) with a superantigen to the TCR of Jurkat T cells. Recruitment of CD31 to the synapse and the downstream signaling of the TCR were analyzed by fluorescence microscopy. Calcium mobilization, CD69 expression and IL-2 production were quantified by cytometry.

Results: We found that the CD31 molecule is consistently recruited to the synapse.In the pSMAC area the CD31 molecule is intact and co-localizes with ICAM1, while a cleaved form clusterizes with the TCR complex in the cSMAC. The lack of the trans-homophilic membrane-distal Ig domain of CD31, lost with the cleavage, favors synapse formation as demonstrated by cytometric analysis of the Jurkat-RAJI conjugates. Rescue of the ITIM signaling downstream the truncated CD31, with the CD31 peptide, inhibits conjugate formation, calcium mobilization, IL-2 production and CD69 expression, MTOC docking, clusterization of the TCR complex and recruitment of pZAP70 at the immunological synapse via an increased recruitment of pSHP2.

Conclusions: We concluded that CD31 acts as an important co-inhibitory receptor involved in the regulation of adaptive immune responses by negatively affecting the formation of the immunological synapse.

P0588

The PI 3-kinase/Akt signalling axis regulates integrin-mediated lymphocyte adhesion and motility

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Purpose/Objective: In the immune system, integrin-mediated adhesion is important for lymphocyte homing, activation and effector functions. The PI3-kinase pathway has been previously implicated in the regulation of lymphocyte homing. We have now investigated the roles of PI3-kinase and two downstream kinases, PDK1 and Akt in integrin-mediated functions in lymphocytes.

Materials and methods: Splenic B cells and effector T cells generated from wild-type mice were treated with PI 3-kinase inhibitors (LY-29004 and GDC-0941) and Akt inhibitors (Akt VIII inhibitor and MK-2206). PDK1-deficient cells were generated using Cre/Lox methodology with Tamoxifen treatment. Cell adhesion to ICAM-1 ligands was assessed using solid-phase assay with or without introduction of shear flow. TIRF microscopy was used to observe 2D motility and Atomic Force Microscopy was used to assess strength of integrin-ligand unbinding force.

Results: Effector T cells have high PI 3-kinase/Akt activity, and the pre-treatment of these cells with PI 3-kinase inhibitors significantly reduced effector T cell adhesion to ICAM-1. This suggests a role for PI3-kinase in regulating aLb2 integrin-mediated adhesion. Both PDK1-deficient effector T cells and Akt inhibitors-treated effector T cells showed reduced adhesion to ICAM-1. Akt inhibitor-treated effector T cells exhibited significantly reduced ability in 2D motility on ICAM-1 than untreated cells. Measurements using Atomic Force Microscopy also showed that the force required for unbinding of integrin-ligand interactions was significantly lower for Akt inhibitor-treated effector T cells than untreated cells. However, Akt inhibition does not affect effector T cells adhesion to ICAM-1 under shear flow. In both unstimulated and BCR-stimulated naïve murine B cells, PI 3-kinase and Akt inhibitors significantly reduced binding to integrin ligands. Additionally, Akt inhibitors reduced SDF-1-induced B cell adhesion to ICAM-1 under shear flow. In contrast, integrin-mediated adhesion of $CD4^+$ T cells was not affected by Akt inhibition either under static and shear flow conditions.

Conclusions: With these results, we propose that the PI 3K/PDK1/Akt signalling axis plays a role in the regulation of lymphocyte integrin function and homing, but the adhesion of different lymphocyte subsets (naïve B cells and effector T cells) are differently dependent on Akt.

P0589

The role of protein tyrosine phosphatase PTPN22 in T cell signaling

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Purpose/Objective: A dynamic balance between tyrosine phosphorylation and dephosphorylation is crucial for the maintenance of homeostasis and proper regulation of immune system. A large number of protein tyrosine phosphatases (PTPs) are known to regulate signaling through the T cell receptor (TCR). The protein tyrosine phosphatase PTPN22 (also called PEP/LYP) is a negative regulator of T-cell activation. An allelic variant of PTPN22, a missense C1858T ($PTPN22^{R620W}$) single nucleotide polymorphism, is associated with multiple autoimmune diseases in humans. Studies conducted with human cells indicated that this mutation was a gain of function leading to decreased TCR signaling. By contrast, studies conducted in a comparable knock in mouse model, $PTPN22^{R619W}$, suggested this variant was a loss of function, as the $PTPN22^{R619W}$ protein was more prone to calpain mediated degradation. Thus the $PTPN22^{R619W}$ mouse was phenotypically similar to the PTPN22 KO mice and both showed an increase in T effector/memory cells. Whether the allelic variant is a loss- or gain-of function still needs to be resolved. In order to address this question we have examined the role of $PTPN22^{R619W}$ *in vitro* and *in vivo* using retroviral and lentiviral transduction.

Materials and methods: Lentivirus and retrovirus expression constructs were derived by subcloning full length wild type *Ptpn22* (619R) or mutant *Ptpn22* (619W) cDNAs containing C- terminal One-Strep tag and an EGFP reporter gene. $PTPN22^{-/-}$ T cells and bone marrow cells were transduced *in vitro* by retroviral generated supernatant. FACS analysis was conducted to confirm the expression of PTPN22 and functional assays performed on transduced cells.

Results: naïve T cells were recovered from Rag-KO chimeras reconstituted with $PTPN22^{-/-}$ bone marrow transduced with vector alone, $PTPN22^{WT}$ and $PTPN22^{R619W}$ retroviral constructs. Preliminary studies indicate that retroviral expression of either construct reduced T cell activation compared to $PTPN22^{-/-}$ T cells. WT PTPN22 appeared to be more suppressive of T cell responses than variant $PTPN22^{R619W}$.

Conclusions: Our data point to the variant $PTPN22^{R619W}$ as being a partial loss of function allele. Further studies are underway to clarify how the WT and mutant alleles influence T cell signaling.

P0590

TRAIL receptor co-stimulation inhibits human T cell proliferation by preventing proximal T cell receptor signalling

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Purpose/Objective: Death receptors are originally characterized as apoptosis inducers but are now increasingly recognized as surface receptors with additional functions depending on cell type, stimulation, and cellular environment. Activation of TRAIL receptors e.g. can either induce apoptosis or proliferation in tumor cells depending on the tumor phenotype. In this study, we want to elucidate the role of TRAIL receptor co-stimulation during T cell activation.

Materials and methods: To analyze the effect of TRAIL receptor co-stimulation on T cells, CFSE-labeled human T cells from buffy coats of healthy donors were stimulated with anti-CD3 and -CD28 antibodies in the absence or presence of recombinant human TRAIL. T cells were analyzed for proliferative capacity, expression of activation markers, and cytokine secretion. In order to define the effects of TRAIL receptor co-stimulation on T cell signal transduction, tyrosine phosphorylation, MAP kinase and caspase activation, and nuclear translocation of transcription factors were analyzed.

Results: CD3/CD28 triggered activation of T cells is inhibited by TRAIL receptor co-stimulation in a dose-dependent manner. This effect is dependent on TCR activation, since no inhibition of T cell proliferation is observed after activation with PMA and Ionomycin. Inhibition of T cell proliferation by TRAIL receptor co-stimulation can not be reverted by IL-2 and is only achieved by immobilized TRAIL. TCR and TRAIL receptor stimulation leads to decreased expression of activation markers CD25, CD69, CD71, CD95, and HLA-DR compared to TCR stimulation alone. The secretion of cytokines IL-2, IFN-γ, TNF-α, IL-5, and IL-13 is efficiently suppressed after TRAIL receptor co-stimulation. T cell activation in the presence of TRAIL receptor triggering efficiently inhibits the phosphorylation of tyrosine and MAP kinases and blocks nuclear translocation of transcription factors NFAT, NFκB, and AP-1.

Conclusions: Co-stimulation of TRAIL receptors in T cell activation specifically inhibits TCR mediated T cell proliferation and activation by blocking the induction of TCR proximal pathways such as tyrosine phosphorylation, MAP kinase activation, and translocation of transcription factors. Althogether, we could show that TRAIL receptors not only act as death receptors, but have an important function in immunoregulationof T cells.

Poster session: Lymphocytes in Tissues

P0591

Cell depletion and regulatory T cell in thymus of alloxan-induced diabetic mice

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Purpose/Objective: In this work we investigated cell death in CD3+ cells, expression of apoptotic proteins and the presence of regulatory T cell in thymus of alloxan-induced diabetic mice.
Materials and methods: Expression *in situ* of apoptotic and anti-apoptotic proteins was detected through immunohistochemical stain protocol and gene expression of apoptotic proteins was detected by real time polymerase chain reaction technique. To evaluate apoptotic cells, was performed Tunel stain *in situ* and flow cytometry for Annexin-V in total thymus cellularity. Regulatory T cells were observed by flow cytometry using FoxP3 Staining kit from E-Bioscence.
Results: It is possible to observe that thymus from aloxan-induced diabetic mice presents an increase of apoptotic cells when compared to non-diabetic animals. Cell death in diabetic thymus probable is higher due to an increase in apoptotic protein levels as loss of survival cytokines expression, such IL-2. Diabetic thymus also presents alterations in expression of apoptotic molecules genes. Also, was observed an altered pattern in the regulatory T cell population in atrophic thymus.
Conclusions: Atrophic thymus from diabetic mouse presents a deep commitment in thymocyte population which is activated for cell death and to express a regulatory phenotype.

Ethical approval: All experiments were approved by the internal ethical committee (CEUA #2312-1).

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P0592

Central memory CD8 T cells regulate hematopoietic stem cell function

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Purpose/Objective: After immune activation, effector T cells, including virus-specific CD8 T cells, are known to migrate to the bone marrow (BM), but their function at this site is unknown. Since BM T cells are known to have a positive influence on engraftment of hematopoietic stem cells (HSCs) upon allogeneic transplantation, we here test the hypothesis that T cells present in the BM can directly influence the function of hematopoietic stem cells (HSCs).
Materials and methods: Highly purified HSCs from murine or human origin were cocultured together with different subsets of BM T cells to determine their capacity to adjust the function of the HSCs.
Results: We found that murine BM T cells can enhance the differentiation capapcity of HSCs as well as their ability to self-renew. This feature is specific for BM CD8 central memory T (T_{CM}) cells, since other T cell subsets are not able to affect HSCs. Moreover, depletion of CD8 T_{CM} cells from the total BM T cell pool abrogates their impact on HSC differentiation and self-renewal. *In vitro* studies revealed that BM CD8 T_{CM} cells do not affect quiescence of HSCs, but do enhance their proliferative capacity. We also found that supernatant from stimulated and non-stimulated CD8 T_{CM} cells is sufficient to increase HSCs numbers *in vitro*. Competitive transplantation assays performed in irradiated mice showed that HSCs cultured with CD8 T_{CM} cell-derived supernatant perform much better in multilineage reconstitution than medium-treated HSCs. Finally, we could demonstrate that adoptive transfer of BM CD8 T_{CM} cells is sufficient to restore the function of HSCs in T cell-deficient mice. Preliminary data indicate that these findings are also relevant for the human situation, since we found that autologous BM T cells can increase the numbers of human HSCs, as well as their differentiation capacity *in vitro*.
Conclusions: Overall, these findings demonstrate that T cells have an important function in the BM and that especially CD8 T_{CM} cells can directly influence HSC homeostasis. We postulate that this feedback mechanism of the immune system on the hematopoietic process in the BM is particularly relevant during viral infection, as the efficient migration of virus-specific CD8 T cells to the BM could well benefit the replenishment of the HSC/progenitor cell compartment and restoration of blood cell numbers that got lost upon infection.

P0594

Characterization of T cells in the distal lung A Hutton, JA Warner and C Pickard, Faculty of Medicine, University of Southampton

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Purpose/Objective: To investigate the CD4+ve and CD8+ve T cell populations in the distal lung of patients with COPD.
Materials and methods: Tissue was obtained from patients undergoing resection for carcinoma at Southampton General Hospital.Patients were classified as no evidence of airways obstruction (FEV1/FVC = 0.78 ± 0.02) or as mild/moderate COPD (FEV1/FVC = 0.57 ± 0.02). T cells were isolated from the distal lung by mechanical and enzymatic disruption followed by red cell lysis. Resultant cells were stained for CD3, CD4 and CD8 and analyzed via flow cytometry. Dead and apoptotic cells were excluded from analysis following 7-AAD and Annexin V staining.
Results: We found substantial numbers of CD4+ve T cells in the distal lung of patients with (median = 40.7% of total CD3+ve cells) and without (median = 36.4% of total CD3+ve cells) COPD. The proportion of CD8+ve cells in the two groups was also similar with a median of 43.1% in the no COPD group and 38.8% in the COPD group. We found no relationship between the proportion of CD4+ve or CD8+ve cells and patient lung function. There were also substantial numbers of CD3+ve, CD4-ve, CD8-ve cells in both groups (median 6.3% for no COPD and 7% for the COPD patients) and CD3+ve, CD4+ve CD8+ve cells (median 5.2% for no COPD and 4.5% for the COPD patients).
Conclusions: A substantial percentage of the CD3+ve cells isolated from the distal lung were CD4+ve with a similar percentage of CD8+ve cells. We could also identify populations of double positive and double negative cells. We found no evidence that any of the populations were altered in mild/moderate COPD. There was no relationship between number of CD4+ve or CD8+ve cells and lung function. Our future research will aim to explore the antigen specificity of these T cells; in particular examining carbonyl modified self-proteins as potentially stimulatory antigens.Thus we are aiming to investigate a possible autoimmune aspect to COPD.

P0595

Characterization of the renal CD4(+) T-cell response in experimental autoimmune glomerulonephritis

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Purpose/Objective: Anti-glomerular basement membrane glomerulonephritis results from an autoimmune reaction against the Goodpasture antigen, the non-collagenous domain of the alpha3 chain of type 4 collagen (alpha3IV-NC1). The production of autoantibodies and their deposition along the basement membrane of glomeruli and pulmonary alveoli are central to pathogenesis of disease. However, there is also increasing evidence from clinical and experimental data for a contribution of T cells to disease development.

Materials and methods: We use the mouse model of experimental autoimmune glomerulonephritis (EAG) to analyze the role of T cells in renal destruction.

Results: Following immunization with recombinant alpha3IV-NC1, DBA/1 mice display a pathogenesis that reflects all hallmarks of human anti-glomerular basement membrane glomerulonephritis. We observe an initial alpha3IV-NC1-specific IgG response with deposition of autoantibodies along the glomerular basement membrane and progressive proteinuria. In this stage, the kidney shows only marginal signs of inflammation. After 9–13 weeks, mice develop severe signs of glomerulonephritis including crescent formation as well as massive tubulointerstitial damage with accumulation of T cells and macrophages. Renal inflammation eventually results in a severe loss of kidney function accompanied by massive edema formation. CD4+ T lymphocytes isolated from the kidneys of end stage EAG mice display a highly activated phenotype and produce TNFalpha, IFNgamma and IL-17A upon polyclonal *in vitro* stimulation. Using highly sensitive FACS-based techniques, we can also detect renal CD4+ T cells that produced cytokines in response to stimulation with alpha3IVNC1.

Conclusions: From our data, we conclude that renal accumulation of autoreactive T cells might play a role in the progression from mild renal inflammation to severe glomerulonephritis with cell-mediated destruction of glomerular structures and severe tubulointerstitial inflammation.

P0596

Functional properties of resident B cells in arterial walls of human atherosclerotic patients

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Purpose/Objective: Animal models suggest that B cells may have contradictory protective or pro-atherogenic effects. To further understand the pathophysiology of human atheroma, we analyzed B lymphocytes present in vascular lesions.

Materials and methods: Ig repertoires were analyzed by RT-PCR on whole tissue extracts from carotid endarterectomy samples of 13 patients using adapted V and C primers. Cytokine, surface marker and transcription factor mRNA expression was studied on adventitial lymphocytes isolated by laser capture micro dissection (LCM).

Results: In adventitia, B lymphocytes constituted diffuse infiltrates or formed small cell clusters adjacent to plaques and BCR sequence analysis revealed that individual samples contained each a limited number of B cell clones. Functional $\alpha 1$, $\alpha 2$, $\gamma 1$ and $\gamma 2$ mRNAs comprised the majority of adventitial Ig H chain mRNAs. In some samples, IgA was expressed as the only H chain isotype. IgM was absent in many adventitias. Most L chain transcripts were of the λ type. V regions of all γ, α and one micro transcripts were hyper mutated and used recurrent VDJ associations. Moreover, a number of genetically distinct VH and VL regions evolved to eventually code for identical CDRs. Expression of the cytidine deaminase AID was detected in several arterial wall samples, in keeping with the observation of a local H chain class switch. Using CDR3 sequences as clonotypic markers, we found that plaque and adventitia repertoires were different and that some B cells were trafficking between adventitia and draining lymph nodes. Most resident B lymphocytes could be described as CD20 negative plasmablasts. However, they lacked markers of terminal differentiation to plasma cell (CD138 and Blimp-1). LCM isolated adventitial B lymphocytes had an activated phenotype: they expressed IL-6, GM-CSF and TNF-α whereas IL-2, IL-4, IL-10, M-CSF and IFN-γ were not detected.

Conclusions: In atherosclerosis, arterial wall B cells are mainly mature B2 lymphocytes and thus differ from B1 cells responsible for the systemic production of protective antibodies. CDR3 convergence, AID expression, clonal H chain switch and an inverted λ/κ ratio of L chain usage witness for a local and antigen-driven maturation that selects oligoclonal adventitial B lymphocytes able to act on inflammation and disease progression directly or by promoting polarization of other immune cells.

P0597

Gliadin- or secalin-primed $CD4^+$ $CD62L^-$ CD44high T cells as sensors of dietary gluten – practical application of a mouse model of gluten-sensitive enteropathy

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Purpose/Objective: We recently described a model of gluten-sensitive enteropathy, based on the adoptive transfer of gliadin-sensitized $CD4^+$ CD45RBlow $CD25^-$ T cells (FACS-sorted) into $Rag1^{-/-}$ mice. Here, we asked whether a different memory T cell population, $CD4^+$ $CD62L^-$ CD44high T cells, would be equally potent in the induction of enteropathy. We also tested in this disease model the immunostimulatory and inflammatory effects of germinated rye sourdough, a food product from a baking system characterized by extensive prolamin hydrolysis (Loponen J *et al.*, J Agric Food Chem 2009).

Materials and methods: In modification of a published protocol (Freitag TL *et al.*, Gut 2009), $Rag1^{-/-}$ or nude mice were injected with column-prepared, splenic $CD4^+$ $CD62L^-$ CD44high T cells from wheat gliadin- (or rye secalin-) immunized wildtype donor mice. Recipient mice were then challenged with diets containing different doses of gliadin (or secalin), different doses of either germinated or native rye sourdough (10 or 50 g/kg diet), or gluten-free control diet. Prolamin content of mouse diets was reassessed by R5 ELISA.

Results: $Rag1^{-/-}$ recipients of gliadin- (or secalin-) sensitized $CD4^+$ $CD62L^-$ CD44high T cells lost more body weight and suffered from significant exacerbation of histological duodenitis when challenged with diets containing 2.5 g gliadin (or secalin)/kg diet versus

gluten-free diet. Secretion of IFNg by splenocytes in response to secalin restimulation was also increased. In nude mice, serum anti-secalin IgG and IgG2c titers (ELISA) were strongly reduced in the 50 g/kg germinated versus native rye sourdough group. However, we found no difference in body weight development, histological duodenitis scores or splenocyte cytokine secretion between 2 groups of $Rag1^{-/-}$ mice challenged with 50 g/kg germinated versus native rye sourdough.
Conclusions: Column prepared, gliadin- (or secalin-) sensitized $CD4^+$ $CD62L^-$ CD44high memory T cells are effective in the induction of gluten-sensitive enteropathy in lymphopenic mice. Hydrolysis of secalin in germinated-rye sourdoughs remains incomplete. While B cell epitopes appear to be destroyed more efficiently, secalin peptides retain T cell stimulatory capacity. Our results caution against the use of antibody-based prolamin detection methods when testing the safety of processed cereal food products for celiac patients.

P0598

Identification and characterization of CD4+Helios+Foxp3- memory-like T cell population in Peyer's Patches

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Purpose/Objective: The concept of immunological memory has been well recognized for protective immunity to pathogens, however, much less is known about food-specific memory T cells. Tolerance to food antigens affects the responsiveness of the local immune system in the intestinal mucosa itself, but is also manifested by the suppression of immune reactions to subsequent systemic antigen challenge, a phenomenon known as oral tolerance. Most aspects of oral tolerance are dependent on $CD4^+$ T cell immune responses including clonal deletion, clonal anergy and induction of regulatory T (Treg) cells. The question remains whether T cells are able to generate potentially dangerous memory responses following oral uptake of innocuous dietary antigens.
Materials and methods: Adoptive transfer of $CD4^+$ T cells: $CD4^+$ T cells were purified from OT-II mice and were transferred i.p. into C57BL/6 mice. Tolerance induction: Oral and intestinal tolerance was induced by supplementing drinking water with 2 mg/ml OVA. Intracellular cytokine staining (ICS) and flow cytometry: Single cell suspensions were obtained from various organs. The stained cells were analyzed by flow cytometry.
Results: In this study, we show that soluble dietary antigens induced high frequencies of $CD4^+Helios^+$ $CD44^+$ memory T cells in Peyer's Patches (PP). These cells were present in both WT mice and germ-free mice representing the most abundant T cell population in this organ. Apart from the Helios expression, they did not share substantial functional similarities with Treg cells. Although many activation/memory markers were expressed on $CD4^+Helios^+Foxp3^-$ T cells, the immunomodulatory environment within PP led to silencing of IL-7R signalling in this T cell population.
Conclusions: Despite the large extent of antigenic exposure, the intestinal immune system has the unique propensity to evoke hyporesponsiveness to innocuous food antigens. Although the priming of naïve T cells might take place at various sites in the gut, we here demonstrate that the storage of potentially dangerous food-specific $CD4^+$ T cells occurs mainly in PP, where the downregulation of IL-7R signalling provides their dormant and apoptotic behaviour.

P0599

IL-18 regulates effector cells of both the innate and adaptive immune response in models of lung inflammation

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Purpose/Objective: Interleukin-18 has been shown to prime a number of cell types enhancing their response to exogenous and endogenous signals. The ability of IL-18 to induce IFNg production by T cells and NK cells when used in the presence of IL-12 has been well characterised and associates IL-18 with the development of TH1 and CD8 responses. Conversely, a number of studies have suggested that IL-18 could enhance IL-4 and IgE production when in the presence of TH2 promoting signals. We describe a set of *in vivo* experiments to further address the role of IL-18 on effectors of the innate and adaptive immune response in the lung.
Materials and methods: The cellular infiltrate and mediators being present in the lung of mice were characterised in 2 mouse models using IL-18KO mice or a neutralising anti-IL-18 antibody. Firstly, we assessed lung inflammation 24 h after an intranasal LPS challenge. Secondly, we performed subcutaneous immunisation of ovalbumin (OVA) in complete Freund's adjuvant followed by six aerosolised OVA challenges 3 weeks later. This model aimed to assess the contribution of IL-18 to TH1 and TH2-driven lung responses.
Results: In the acute LPS challenge model abrogation of IL-18 signalling resulted in a significant decrease in the total number of cells present in the broncho-alveolar lavage, neutrophils being the main leukocyte population affected. In the OVA lung inflammation model a significant reduction in the number of eosinophils infiltrating the lung was observed in IL-18KO mice with no significant change to the recruitment of neutrophils or T cells. Surprisingly, when antigen-specific responses were analysed using Pentamer staining, an increased frequency of OVA-specific CD8 cells was observed in IL-18KO mice compared to wild type mice. This was associated with an increased production of IFNg upon *ex-vivo* re-stimulation of lung T cells.
Conclusions: In an acute model, we showed that IL-18 plays a role in early inflammatory events leading to lung neutrophilia associated with an LPS challenge. In addition, IL-18 also affects antigen-specific T cell responses as exemplified by the decreased TH2-driven eosinophilia observed in IL-18KO mice. These preliminary findings highlight the complexity of the role of IL-18 which is dependent on the context in which it is released.

P0600

Light, xenobiotics and AHR activation in Lupus patients

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Purpose/Objective: Sun exposure or the ingestion of certain medications are typical triggering factors in the development of autoimmune disease flares. Systemic Lupus Erythematosis (SLE) is often cited as the prototypical potentially photo- or medication-induced autoimmune disease. The immunopathologic effect resulting from exposure to these two seemingly different triggering factors remains, to date, unknown.
Materials and methods: Intracellular IL-17 and IL-22 was detected, gated on CD4+ T lymphocytes, using FACS analysis of PBMC stimulated 18 h with anti-CD3 and anti-CD28 antibodies. Quantitative RT-PCR was applied to unstimulated PBMC and lesional skin of SLE

patients, using primers specific for IL-17, IL-22, Cyp1a1, CD3 or §-actin. Several drugs implicated in the induction of SLE were screened, in their native forms and after UV exposition, for their abilities to activate the Aryl Hydrocarbon Receptor (AHR) and induce Cyp1a1 mRNA expression. Propranolol and its UV-induced derivatives were studied by mass spectrometry. Th17 differentiation was performed on CD4+ cord blood cells using anti-CD3 and anti-CD28 antibodies in the presence of IL6, IL1b and TGFb. Cytokines were evaluated using a bead-based assay (Luminex) and ELISA.
Results: We confirm that during SLE flares the circulating Th17 subpopulation is amplified. This augmentation is not typically found in other systemic auto-immune diseases such as primary Sjögren syndrome or in 'primary' antiphospholipid syndrome. The aryl hydrocarbon receptor (AHR) plays a central role in Th17 differentiation. We found that SLE patients are hyper-responsive to AHR ligands and show characteristic signs of AHR activation in their skin lesions that strongly correlate with lesional interleukin-22 levels. Finally, we show that the photo-induced degradation products of certain medications are equally capable of activating AHR *in vitro*.
Conclusions: Taken together these results allow us to reconcile the patho-physiologic effects of medications and UV light by relating the two mechanisms to local activation of Th17 cells.

P0601
Lymph node dissection – understanding the immunological function of lymph nodes

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Purpose/Objective: Lymph nodes (LN) are one of the important sites in the body where immune responses to pathogenic antigens are initiated. This immunological function induced by cells within the LN is an extensive area of research. To clarify the general function of LN, to identify cell populations within the lymphatic system and to describe the regeneration of the lymph vessels, the experimental surgical technique of LN dissection was established in various animal models.
Materials and methods: For dissection of the mesenteric LN (mLN) the abdomen was opened and the gut was taken out so that the mLN were seen. The mLN were excised carefully in order not to injure the superior mesenteric artery lying behind, whereas the connection of the lymph vessels and small blood vessels to the LN was disturbed. Afterwards the gut was replaced in the abdomen and the abdomen was closed.
Results: To analyze the different cell subsets of the incoming lymph in detail LN were dissected. Furthermore, LN were identified as the place where the induction of an antigen-specific response occurs and more significantly where this immune response is regulated. During bacterial infection, LN, as a filter of the lymph system, play a life-saving role. In addition, LN are essential for the induction of tolerance against harmless antigens, because in LN-resected animals tolerance could not be induced.
Conclusions: The technique of LN dissection is an excellent and simple method to identify the important role of LN in immune responses, tolerance and infection.

P0602
RGS-1 in intestinal T cell trafficking and responsiveness

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Purpose/Objective: Few clear mechanisms have been defined by which the regulation of gut T cell responses may be distinguished from systemic T cell regulation. In addition, how lymphocytes selectively respond to, or ignore, chemotactic signals in the gut is very poorly understood. Regulator of G protein signalling 1 (RGS1) might be a key regulator of T cell trafficking in the gut based on its conspicuously high expression in gut-associated T cells.
Materials and methods: We have evaluated the role of RGS1 in human and mouse T cell migration using transfected and knock-out cells, respectively. The colitogenic potential of RGS1-deficient T cells was evaluated in $CD4^+$ $CD45RB^{Hi}$ T cell transfer model of colitis.
Results: We have shown that RGS1 is elevated (50–100-fold) in human (and mouse) gut T cells compared to peripheral T cells. Consistent with its capacity to regulate the $G\alpha_i$ subunit of GPCRs, RGS1 inhibited the activity of specific chemokine receptors, reducing human and mouse T cell migration to selected cytokines, e.g. CCL19, CXCL12, but not to gut-homing chemokines, CCL25 and CCL20. In addition, RGS1 levels are further elevated in T cells derived from inflamed gut and the colitogenic potential of RGS1-deficient T cells was significantly reduced, Interestingly, GWAS strongly implicate the RGS1 locus in celiac disease, multiple sclerosis and Type I diabetes. RGS1 is also highly expressed by regulatory T cells. We therefore explored further its regulation of T cell responsiveness, finding that RGS1-deficient cells make exaggerated cytokine responses upon activation *in vitro* and *in vivo*.
Conclusions: Our data suggest that RGS1 ordinarily represses T cell egress from the gut, possibly to sustain local immunoprotection and/or immunoregulation vis-a'-vis commensals. Ongoing work aims to further discern to what degree this non-redundant regulation of IEL by RGS1 contributes to the unique state of rapidly responsive body-surface lymphocytes, as well as to what degree differential expression of other RGS molecules regulates intraepithelial T cell subsets with implications for diseases in different tissues. In sum, RGS1 emerges as a novel, site-specific T cell regulator that may prove an effective clinical target, complementing the intense pharmacological targeting of chemokine receptors.

P0603
Role of T-cells in cardiac ischemia-reperfusion injury

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Purpose/Objective: The extent of injury after acute myocardial infarction is determined by duration of ischemia. However, after coronary flow restoration, the myocardium is additionally damaged by reperfusion. This so-called reperfusion injury is partially mediated by recruitment of inflammatory cells. Yet, the role and activation patterns of T-cells (TCs) after myocardial reperfusion have not been established.
Materials and methods: The left coronary artery was surgically occluded in male mice for 30 min (ischemia), followed by 3, 6, 18

or 24 h of reperfusion. FACS analysis and immunohistochemistry were performed to characterize the time course of inflammatory cell infiltration during reperfusion in WT mice. After 24 h of reperfusion, WT, CD4KO, CD8KO and OTII mice underwent Evans Blue/TTC-staining to determine the infarct size in relation to the area at risk. As control, mice underwent a sham operation procedure (sham).
Results: Significantly more CD3+ TCs infiltrated the mouse myocardium after 30 min ischemia and 24 h of reperfusion in comparison to sham. CD4+ TCs (47%) and CD8+ TCs (30%) are the predominant subsets among all $CD45^+$ $CD3^+$ leukocytes recruited to the myocardium during the entire reperfusion period. NK TCs (12%) and gd TCs (5%), but not B lymphocytes could be detected. Evans Blue/TTC-staining demonstrated that CD4 KO and OTII mice, but not CD8 KO mice were partially protected. The reduction in infarct size in OT II mice indicates that antigen recognition is relevant for the harmful effect of CD4+ TCs on ischemia-reperfusion injury.
Conclusions: We could demonstrate that, besides innate immune cells, there is considerable infiltration of TCs into infarcted myocardium after ischemia-reperfusion. CD4+, but not CD8+ TCs contribute to myocardial ischemia-reperfusion injury, obviously after activation by recognition of self-antigens.

P0604
Th17/Tc17 recruitment and positioning in the human hepatic inflammation

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Purpose/Objective: IL-17 secreting CD4 (Th17) and CD8 (Tc17) are distinct lineages of T lymphocytes implicating autoimmunity, bacterial infections in murine models and human diseases. They are implicated in several inflammatory, autoimmune and viral liver diseases and also in progression of hepatocellular carcinoma. However little is known about human Th17 & Tc17 cells molecular recruitment mechanism and positioning in the inflamed human liver.
Materials and methods: Human liver infiltrating (LI) lymphocytes were freshly isolated from explanted liver tissues. LI-Th17&Tc17 cells frequencies, phenotype, chemokine receptors, integrin expression and cytokine secretion assessed *ex-vivo* by flow cytometry. Frequency and distribution in tissue determined using dual immunohistochemistry and confocal microscopy. Recruitment was studied *in-vitro* using IFN-g stimulated hepatic sinusoidal endothelium cells (HSEC) in flow based adhesion assays. Positioning around bile duct was investigated by chemotasis. Chemokine expression and secretion from bile duct was assessed by immunohistochemistry and ELISA.
Results: Inflamed human liver contains a higher proportion of LI-IL-17 cells comprising 3% of T cells infiltrate with associated neutrophils. LI-Th17&Tc17 express RORc, IL-23 receptor and secrete IL-17, IL-22, TNF and IFN-g. $RORc^+$ liver infiltrating $IL17^+$ cells did not co-express $Foxp3^+$. Liver infiltrating Th17 and Tc17 express chemokine receptors CXCR3, CCR6 and integrin LFA-1, VLA-4. Human peripheral blood Th17/Tc17 cells were recruited via inflamed HSEC by CXCR3, ICAM-1, VCAM-1, and VAP-1 dependent mechanisms. IL-17 receptor is expressed on HSEC and IL-17 treatment up-regulates adhesion molecules and chemokines on HSEC creating a paracrine loop to promote further recruitment. Th17 cells were located close to inflamed bile ducts that express the CCR6 ligand, CCL20. Human cholangiocytes stimulated with TNF-α and IL-17 secreted CCL20 and induces CCR6-dependent migration of human Th17 cells.
Conclusions: Th17 and Tc17 cells are recruited via hepatic sinusoidal endothelium using CXCR3, integrin and VAP-1 dependent mechanisms. IL-17 secreted by infiltrating cells has paracrine effects on endothelium leading to enhanced Th17 recruitment. Their subsequent positioning near bile ducts is dependent on cholangiocyte-secreted CCL20.

P0605
The function of the chemokine receptor CXCR6 in the T-cell response against Listeria monocytogenes

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Purpose/Objective: The chemokine receptor CXCR6 recognizes the chemokine CXCL16 which is expressed in a transmembrane form by sinosoidale enothelium cells and in a secreted form by activated dendritic cells. CXCR6 is expressed on NK cells and NKT cells as well as activated CD8 T cells and CD4 Th1 cells. CXCR6 controls accumulation and survival of NKT cells in the liver. However, the function of CXCR6 in conventional T cells is less well understood.
Materials and methods: Here, we use the Listeria monocytogenes infection model to characterize the role of CXCR6 in the regulation of CD8 T cell responses.
Results: Expression studies revealed that particularly in the liver a large fraction of conventional CD4 and CD8 T cells expressed CXCR6. The chemokine receptor was further upregulated on activated CD4 and CD8 T cells during listeria infection. Expression of CXCR6 on listeria-specific T cells was a late event and regulated independently from IFNg expression. Following infection, CXCR6-deficient mice showed similar listeria titers in spleen and reduced titers in liver. Analysis of T-cell responses in CXCR6-deficient mice, revealed only minor changes in frequencies of listeria-specific CD4 and CD8 T cells in spleen as well as in liver. T cells from infected wild type and CXCR6-deficient mice also showed comparable cytokine profiles following *in vitro* restimulation. Listeria-specific T cell responses were also analysed with a competitive T-cell transfer assay. This approach revealed an altered tissue distribution of CXCR6-deficient CD8 T cells, with reduced frequencies in blood, lymph nodes and liver at late time points post infection. At early time points, T cell frequencies were reduced only in the blood. Interestingly, transferred CXCR6-deficient CD8 T cells showed diminished proliferation at these time early points.
Conclusions: In summary, CXCR6 controls tissue distribution of CD8 T cells but has only limited impact on the acquired CD8 T cells response against L. monocytogenes infection.

P0606
The role of the small GTPase Arl4d in CD8 T cell immune function

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Purpose/Objective: The liver is known to induce tolerance rather than immunity. Liver sinusoidal endothelial cells (LSEC) can induce a non-responsive state in naïve CD8 T cells after antigen-specific stimulation. The ADP-ribosylationfactor/ARF-likeprotein (ARF/Arl) family regulates membrane traffic and organelle structure. Arl4d is localized to the plasma membrane and can recruit cytohesin-2/ARNO, where it modulates actin remodeling. We found Arl4d to be overexpressed in LSEC-stimulated CD8 T cells compared to dendritic cell (DC) stimulated T cells in gene expression profiling. Therefore, we further investigated the role of Arl4d in CD8 T cell function.
Materials and methods: Naive CD8 T cells were cocultured with antigen-loaded LSEC or DC. We analysed the kinetics of Alr4d mRNA

and protein expression by real-time PCR and Western Blot, respectively. To investigate the function of Arl4d in CD8 T cells, we used Arl4d$^{-/-}$ mice. CD8 T cells were isolated from the spleen by MACS Cell Separation, which were further stimulated with a-CD3/CD28 antibodies. IL-2 production was analysed by ELISA.

Results: Using real-time PCR and Western blot we could confirm overexpression of Arl4d in CD8 T cells during tolerance stimulation by LSEC *in vitro*. Conversely, Alr4d mRNA was down-regulated in DC-stimulated CD8 T cells. We further found that Arl4d$^{-/-}$ mice do not display overt changes in their lymphoid compartment. Furthermore, we showed that Arl4d is involved in regulating IL-2 production by naïve CD8 T cells. Arl4d$^{-/-}$ CD8 T cells produced significantly more IL-2 than Arl4d$^{+/+}$ CD8 T cells upon TCR stimulation.

Conclusions: Arl4d expression is highly induced during CD8 T cell stimulation by LSEC. In Alr4d$^{-/-}$ mice the lymphoid compartment is not changed and thus Arl4d does not seem to be involved in lymphocyte development. More interestingly, we found Alr4d to be involved in the production of IL-2. As the amount of IL-2 produced by naïve CD8 T cells during antigen-specific interaction with LSEC is an important factor for the induction of non-responsive CD8 T cells, the action of Arl4d may be central to dampen IL-2 production and for subsequent abrogation of immediate effector function in naïve CD8 T cells.

P0607

TNFa acts as a Chemokine to attract CD4+ T cells to rheumatoid joints

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Purpose/Objective: The cytokine TNFα plays a central role in the pathogenesis of rheumatoid arthritis (RA), but its disease-specific effector mechanisms have not been fully elucidated. Aim of the study was the investigation of the role of TNFα in T cell accumulation and migration in synovitic joints of RA patients and the identification of a surface marker which allows discrimination of tissue infiltrating CD4+ T cells from non-infiltrating CD4+ T cells.

Materials and methods: Vital tissue sections from rheumatoid synovium were generated using a horizontally oscillating microtom, and were co-incubated with fluorescence-labelled CD4+ T cells. Migration was detected by fluorescence and confocal microscopy. Migrating T cells were recovered from the tissue using enzymatic digestion and phenotypically analyzed by FACS. Chemokinesis of CD4+ T cells from RA patients and healthy donors in response to TNFα were analyzed in transwell experiments.

Results: CD4+ T cells from RA patients migrated into the tissue sections in significantly higher numbers than CD4+ T cells from healthy controls. Migrating CD4+ T cells differed from non-migrating ones in their increased expression of TNFR1. Furthermore, TNFR1 is expressed on a fraction of circulating CD4+ T cells from RA patients, but not from healthy controls. In a Chemotaxis migration assay, it could be shown that TNFα directly acts as a chemokine for peripheral blood CD4+ T cells from RA patients, but not for CD4+ T cells from healthy controls. Accordingly, blockade of either TNFα or of TNFR1 nearly abrogated *in vitro* T cell migration in synovial tissue.

Conclusions: Taken together, the results indicate, that the interaction of TNFα with TNFR1 is pivotal for T cell migration in synovial tissue *in vitro*, and thereby suggests a relevant role for the cytokine for *in vivo* T cell trafficking to rheumatoid joints.

Poster session: Lymphoid Organogenesis

P0608
Differential regulation of small intestinal and colonic lymphoid tissue development by IL-23 and regulatory T cells

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Purpose/Objective: Isolated lymphoid follicles (ILF) are single B cell follicles found throughout the intestines that develop postnatally and represent a dynamic way in which gut lymphoid system responds to environmental challenges. ILF develop in the colon of transgenic mice lacking genes essential for lymphoid tissue development including small intestinal (SI-) ILF and are less dependent on the intestinal microbiota for their development than SI-ILF. In order to further characterise these differences, the development and maturation of large intestinal (LI-) ILF have been studied in a number of models.
Materials and methods: ILF and mature ILF (mILF) were characterised using wholemount immunofluorescence in mice treated with LTβR-Ig *in utero*. ILF status was also assessed in germ-free mice, IL-23p19$^{-/-}$ mice and mice treated with anti-IL-17, anti-IL-22 or anti-CD25.
Results: LI-ILF develop earlier than SI-ILF, with higher numbers of LI-ILF observed at 2 weeks after birth. Only the colon contained mILF at this timepoint. In germ-free mice, LI-ILF was present and contained mILF in the absence of bacterial stimulation. In contrast to SI-ILF, which increased following colonisation of germ-free mice, LI-ILF were reduced. Interestingly, IL-23p19^{+} cells are enriched in LI-ILF and IL-23p19$^{-/-}$ mice have a reduced number of LI-ILF despite similar numbers of SI-ILF. This phenotype was also present in mice lacking Peyer's patches that had been reconstituted with IL-23p19$^{-/-}$ bone marrow.Antibody neutralisation of IL-22 or IL-17A partially reduced colonic ILF; however neither of these fully recapitulated the phenotype of IL-23p19$^{-/-}$ mice.Foxp3^{+} regulatory T cells (Treg) are also enriched in ILF of both the small intestine and colon and depletion of CD25^{+} Treg increased ILF and mILF in both the ileum and colon.
Conclusions: These results suggest that the homeostatic balance reached with the microbiota through development of ILF in the small and large intestines is differentially regulated by intrinsic factors specific to each microenvironment.The specific regulation of colonic ILF development by IL-23 may have implications in colonic pathology.

P0609
Lymph node transplantation and its immunological significance in animal models

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Purpose/Objective: Lymph nodes (LN) are distributed all over the body and whatever the site consist of the same cell populations. However, there are great differences between LN from different draining areas. For example, in mesenteric LN, homing molecules, e.g. CCR9 and a4b7 integrin, were induced and cytokines, e.g. IL-4, were produced on higher levels compared to peripheral LN.
Materials and methods: To study the immunological functions of mLN and pLN, we established a surgical technique, removing the mLN and transplanting a pLN or another mLN into the mesentery. Briefly, mice were anesthetized and the abdomen opened. The gut was taken out so that the mLN were seen. The mLN were removed carefully not injuring the blood vessels lying behind, whereas the connection of the lymph vessels to the LN was disturbed. Previously excised mLN or pLN from a donor animal were transplanted into this vacant area. After this the gut was replaced carefully in the abdomen and the abdomen closed.
Results: We analyzed the regeneration of the transplanted LN (LNtx).To identify the regeneration of LNtx a kinetic study was performed and the architecture of the LNtx was analyzed. Over a period of 8 weeks a fully regenerated LN developed with connections to lymph and blood vessels. All vessels were shown to be functional, transporting lymph fluid or lymphocytes into the transplanted LN. Furthermore, we could clearly show that lymphocytes from the donor LN disappeared from the LNtx and lymphocytes from the recipient repopulated the transplanted LN. In addition, DC from the draining area migrated from the gut via the afferent lymphatics into the LNtx. Furthermore, using transgenic mice which express the GFP gene in all cells, we showed for the first time that stromal cells remained in the tissue during regeneration. Stromal cells are non-hematopoietic cells, which form the skeletal backbone of the LN by forming a network and extracellular matrix components.
Conclusions: The role of stromal cells as a central cell population within the LN has to be elucidated. In addition, all cell types (stromal cells, lymphocytes and DC) involved in the induction of an immune responses or tolerance concerning foreign Ag or self Ag have to be studied in combination with each other. This could be done by transplanting LN into different draining areas. Therefore, LN transplantation is an important method to examine all these questions.

P0610
M1 inflammatory macrophages act as lymphoid tissue inducer cells in atherosclerosis-associated lymphoid neogenesis

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Purpose/Objective: Atherosclerosis is a chronic inflammatory disorder that is characterized by the formation of tertiary lymphoid structures (TLO) within the adventitial layer. However, the cellular and molecular mechanisms orchestrating TLO formation are still to be investigated. Smooth muscle cells (SMC), that constitute the medial layer of the aorta, have been implicated as chemokine-expressing cells, allowing the formation of TLO. Yet the inducer signal that is locally generated and that confers to SMC their organizer potential is still to be understood. Several lines of evidence suggest that it could be mediated by signaling through TNF- and LT$-receptors, as it is the case during the formation of secondary lymphoid organs.

Herein, we hypothesized that macrophages infiltrated in the plaque could trigger the LT$R/TNFR signaling in vascular SMC, which in turn are responsible for the formation of atherosclerosis-related TLO in the adventitia.
Materials and methods: We analyzed by RT-qPCR the expression of chemokines by mouse primary SMC stimulated by macrophage supernatants and by recombinant LT-α and TNF-α, and their concentration was assessed by ELISA. Matrigel scaffolds loaded with SMC stimulated or not with macrophage supernatant were implanted sub-cutaneously in mice and analyzed by flow cytometry and immunohistochemistry 15 days later.
Results: We found that M1, but not M2 *in vitro*-polarized macrophages induced the expression of TNF-α and LT-α. Moreover, stimulation of SMC with M1-conditioned medium induced the expression of CCL19, CCL20 and CXCL16 chemokines. Importantly, our results showing that stimulation of SMC with rLT-α and rTNF-α induced the same expression profile suggested that M1 macrophages could be responsible for the LT$R/TNFR-mediated triggering of chemokine expression in SMC. This was corroborated with the increased concentration of the three chemokines in the supernatant of rLT-α/rTNF-α-stimulated SMC as compared to unstimulated cells.

Finally, scaffolds loaded with *ex vivo* stimulated SMC presented inflammatory infiltrates that were organized in follicles composed of T and B lymphocytes.
Conclusions: Together, these results suggest that M1 macrophages infiltrated within atherosclerotic lesions are able to trigger the expression of CCL19, CCL20 and CXCL16 by vascular SMC through LT$R/TNFR signalling, allowing the formation of aortic TLO.

P0611
MAdCAM-1 independent lymphocyte homing to GALT of Nkx2.3$^{-/-}$ mice

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Purpose/Objective: Nkx2-3 transcription factor is involved in the regulation of mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expression, and its sequence variants have been identified as susceptibility trait for inflammatory bowel diseases. Although mice lacking Nkx2.3 transcription factor have no endothelial MAdCAM-1, Peyer's patches (PP) and mesenteric lymph nodes (mLN) still develop. As the homing of lymphocytes to gut-associated lymphoid tissues (GALT) is dependent on the high endothelial venules (HEV) displaying MAdCAM-1, we investigated the molecular components of homing to the GALT in Nkx2.3$^{-/-}$ mice.
Materials and methods: The phenotypic features of PP HEVs (PNAd and MAdCAM-1 addressins, production of CCL21 and CXCL13 chemokines) in Nkx2-3 and wild-type BALB/c mice were studied by immunofluorescence. The kinetics of homing to GALT was tested by adoptive cell transfer using CFSE-labeled or GFP/MHC alloantigen-marked donor lymphocytes, followed by flow cytometry or tissue immunofluorescence. The expression of mRNA for addressin proteins and the posttranslational glycosylation enzymes was determined by qPCR. The involvement of MAdCAM-1 or PNAd adressins in the GALT homing was studied *in vivo* using antibody-mediated blockade.
Results: PPs and mLNs of mutant mice showed an enhanced staining for luminal MECA79 epitope against PNAd sulfoglycoepitope, and also increased production of mRNA for several PNAd backbone proteins and modifying enzymes. Adoptively transferred lymphocytes could effectively home to PNAd-positive GALT HEVs, which process could be blocked by MECA-79 anti-PNAd mAb injection, but not with anti-MAdCAM-1 mAb. Although the gut and GALT in Nkx2.3 deficient mice at neonatal age contain MAdCAM-1-positive vessels, in the gut mucosa with organized lymphoid tissues HEVs gradually replace MAdCAM-1 with PNAd, whereas in the non-lymphoid segments of intestinal vasculature the loss of MAdCAM-1 is not coupled with the induction of PNAd.
Conclusions: Together these data indicate that in the absence of endothelial MAdCAM-1 in Nkx2.3$^{-/-}$ mice PNAd controls homing to GALT, thus HEV function is maintained, although with different adhesion molecule expression patterns.

P0612
Presence of lymphoid tissue organizer cells in human tonsil

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Purpose/Objective: Stromal cells from secondary lymphoid organs (SLO) are a heterogeneous population of non-hematopoietic cells. These cells improve survival and differentiation of lymphocytes, are involved in peripheral tolerance and produce the segregation of T and B lymphocytes in different lymphoid areas. In adult SLO, three main types of stromal cells have been identified: follicular reticular cells (FRC) located in the T zone, follicular dendritic cells (FDC) in lymphoid follicles and marginal reticular cells (MRC) detected in the peripheral zone of the lymphoid tissue. During embryonic development, these stromal populations derived from a common precursor called lymphoid tissue organizer (LTo) cells. Our objective was to obtain lymphoid stromal cells from human tonsil and to study the presence of the above mentioned populations.
Materials and methods: Human lymphoid stromal cell lines were isolated from human tonsil using EMEM culture medium with a low concentration of fetal calf serum (3%). Tonsil samples were obtained from patients (3–10 years old) who underwent tonsillectomy for recurrent tonsillitis. Cell phenotype was studied by RT-PCR, flow citometry and histochemistry. Cell lines were differentiated after incubating with the corresponding media.
Results: Human Tonsil Stromal Cells (hTSC) showed to be a homogeneous population that expressed podoplanin and mesenchymal markers (CD10, CD29 and CD73), without contamination by endothelial cells or leukocytes (CD31 and CD45 negative). Moreover, hTSC expressed TRANCE (MRC marker), CXCL13 (MRC and FDC marker), CCL19 and CCL21 (FRC markers), but they were negative for CD21 and CD35 (FDC markers). This phenotype was compatible either with a MRC and FDC mixture, or with LTo cells. The possibility of a different cell types mixture was rejected since hTSC clones also expressed TRANCE, CCL19 and CCL21, but not CD21, CD35 and CXCL13. Finally, hTSC expressed markers associated with mesenchymal stem cells (STRO-1 and CD271) and embryonic stem cells (OCT-3/4 and NANOG). Under appropriate differentiation conditions, they showed adipogenic, chondrogenic and osteogenic potential.
Conclusions: Our results show the presence of LTo-like cells in human tonsil. These cells exhibited a high plasticity, and can be differentiated *in vitro* into other mesenchymal cell types.

P0614
TGF-beta (TGFb) negatively regulates the establishment of the thymic medulla

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Purpose/Objective: Thymic epithelial cells (TEC) are an essential component of the thymic microenvironment. They provide the structural and functional requirements for efficient T cell development. Stochastic rearrangement of the T cell receptor during T cell development necessitates mechanisms to harness potentially auto-reactive T cell clones. Connected with their unique capacity to express peripheral tissue antigens [a.k.a. promiscuous gene expression (pGE)] medullary (m) TEC drive the elimination of auto-reactive T cells clones or foster their development into regulatory T cells. Thus, inborn or acquired numerical or functional mTEC deficiency is linked with autoimmunity. Insight into processes regulating mTEC proliferation and maturation may therefore provide novel directions for therapeutic interventions to improve thymic function.
Materials and methods: We use murine models of TEC-specific inactivation of molecules involved in TGF-beta-signaling and downstream targets to identify the contribution of this pathway to TEC development *in vivo* and *in vitro*.
Results: Genetic abolition of TGFb-signaling specifically in TEC enhances their proliferative potential as well as their terminal differentiation. The ensuing surge in total and mature mTEC is associated with significant increases in both pGE and production of regulatory T cells. In addition, *in vitro* experiments show that TGFb directly modulates alternative NFkB-signaling in TEC. The TGFb target gene c-myc is essential for maintaining the proliferative

but not the differentiation potential of mTEC. In view of a potential therapeutic application of these findings, we show that systemic administration of a small molecule inhibitor of TGFb-signaling alone or in conjunction with Keratinocyte Growth Factor (KGF) results in significant expansion and maturation of mTEC and enhances the thymopoietic effects of KGF.
Conclusions: TGFb negatively impacts the post-natal formation of the thymic medulla in a TEC-autonomous manner and involves the modulation of alternative NFkB signaling. Down-regulation of c-myc expression by TGFb limits the expansion but not the differentiation of mTEC. Our findings that short-term inhibition of TGFb-signaling positively affects the mTEC compartment have implications for therapies aimed at improving thymic function.

P0615
WNT/CTNNB1 pathway inhibition guides the human thymic involution process

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Purpose/Objective: To analyze mRNA and microRNA expression differences between elderly and young human thymuses.
Materials and methods: Four elderly human thymuses (70 years old) and four young thymuses (younger than 10 months) were obtained from individuals undergoing cardiac surgery. mRNA and microRNA differential expression levels were analyzed by microarrays. Expression levels from selected genes was confirmed by quantitative PCR using thymus samples from neonates, 50-years old and 70-years old individuals, all of them obtained from cardiac surgery interventions.
Results: The mRNA analysis showed a great increase in the WNT pathway inhibitors. Results were confirmed by quantitative PCR. Results showed that WNT inhibitors are being mostly produced by non-adipocytic cells. Moreover, both non-adipocytic cells and adipocytes showed a significant increase in adipocyte-differentiation transcription factors mRNA expression levels. The micro-RNA analysis showed changes in miRNAs related with human ageing, stress-related thymic involution, epithelial to mesenchymal transition and control of the WNT pathway. WNT pathway inhibition is compatible with the main processes observed in human thymus: 1) arrest of thymocyte maturation, 2) loss of the cortico-medular structure of thymic epithelial cells and 3) replacement of the epithelial mesh to adipose tissue.
Conclusions: Our results strongly suggest that WNT/CTNNB1 pathway inhibition could be one of the main mechanisms of the human thymic involution process. To stablish this mechanisms is necessary to identify therapeutical targets aiming to slow or even increase thymic function in both, elderly individuals and pathologies associated with lymphopenia and immunological impairment.

Poster session: Lymphomas

P0618
Influence of sphingolipids on lymphoma cell proliferation and survival

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Purpose/Objective: Lipids are important structural membrane components that determine membrane stability and fluidity. Some lipid metabolites also act as signalling molecules by binding and activating specific G protein-coupled receptors on the cell surface, or as cofactors and adapter molecules within cells. Here we evaluated the impact of sphingolipids on lymphoma cell proliferation.
Materials and methods: Different germinal-center-like (GC) and activated-B-cell-like (ABC) diffuse large B cell lymphoma (DLBCL) cell lines were incubated with the naturally occurring D-erythro-Sph (Sph), its unnatural synthetic stereoisomer L-threo-Sph (Lt-Sph), the phosphorylated metabolite sphingosine 1-phosphate (S1P), and its close relative sphingosylphosphorylcholine (SPC).
Results: Of this group of lipids, only Sph and Lt-Sph negatively influenced the proliferation of DLBCL cell lines in a concentration-dependent manner, with Sph having a greater impact than Lt-Sph on GC-, but not ABC-DLBCL proliferation. Since ABC-DLBCL were shown to be significantly more dependent on protein kinase C (PKC) signalling than GC-DLBCL, we investigated the influence of Sph and Lt-Sph on PKC activation. Correspondent results not only confirmed PKC inhibition by Sph, but also revealed a similar activity of its synthetic stereoisomer Lt-Sph. Our data suggest that both Sph and Lt-Sph act as PKC inhibitors by binding to the regulatory C1-domain and competing with endogenous PKC activators like phosphatidylserine (PS). Indeed exogenous application of PS to ABC-DLBCL partially rescued the anti-proliferative effect of Lt-Sph, but not Sph, while it had no effect on GC-DLBCL. Annexin-V and propidium iodide staining with subsequent flow cytometric analysis of cell populations demonstrated cell death, partially induced by apoptosis, and impaired proliferation by a G1-phase cell cycle arrest as the predominant reasons for the observed Sph- and Lt-Sph-induced anti-proliferative effect. Electron microscopy analysis of DLBCL cell lines suggested autophagy as an additional PKC-independent mechanism of Sph.
Conclusions: Thus a cellular increase of Sph potentially interferes with lymphoma growth by inducing cell cycle arrest and cell death via PKC inhibition and autophagy.

P0620
Malignant lymphoma B cells induce dysfunctional cytotoxic T cells

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Purpose/Objective: B cell Non-Hodgkin lymphoma (NHL) is the fifth most common cancer in humans. In several types of cancers, cytotoxic T lymphocytes (CTLs) are commonly found to be dysfunctional and exhausted. However, little is known about how malignant cells modulate the effector function of CTLs. Therefore, we aimed to identify and characterise the mechanism of CTL dysfunction in a murine model of NHL.
Materials and methods: To investigate CTL dysfunction, we used Eμ-Myc transgenic mice that constitutively express the myc oncogene in the immunoglobulin gene locus under the potent Igh locus enhancer (Eμ) on C57BL/6 background. These animals develop B cell lymphoma. We analysed the expression pattern of three inhibitory receptors and absolute numbers of T cell subsets. Furthermore, IFN-γ and TNF-α production from host T cells and adoptively transferred naïve $CD8^+$ TCR transgenic (p14) T cells specific for the gp-33 antigen of lymphocytic choriomeningitis virus (LCMV) in lymphoma-bearing mice were analysed after LCMV infection. The direct impact of malignant B cells on CTL proliferation, apoptosis and cytotoxicity was investigated in co-culture and transwell experiments. Finally, to characterise the soluble factor responsible for T cell dysfunction, supernatants from malignant B cells were fractionated by filter size exclusion and proliferation assays were performed.
Results: Numbers of $CD8^+$ and $CD4^+$ T cells of lymphoma-bearing mice and their ability to secrete IFN-γ and TNF-α were significantly reduced. Activation of specific p14 CTL with LCMV was impaired in lymphoma bearing mice resulting in a reduced cytokine production. The impaired CTL function could be reversed after transfer into secondary C57BL/6 recipients.

Supernatants of lymphoma B cells directly inhibited the proliferation of CTLs *in vitro*, induced apoptosis and reduced the cytokine production of CTLs. Fractionation of cell supernatants revealed that the suppressive factor is <3kDa.
Conclusions: These data demonstrate that dysfunction of CTLs in our NHL model is mediated by a soluble lipid or small metabolite of less than 3kDa secreted by lymphoma cells. Further investigation will focus on the identification of this soluble mediator involved in suppression of CTL function. Finally, these results may provide a therapeutic strategy to enhance immunogenicity against lymphoma.

P0621
MIF expression in leukemic cells

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Purpose/Objective: Invariant chain (Ii) or CD74 is a non-polymorphic glycoprotein whose initial role in antigen presentation was recognized two decades ago. Apart from its role as a chaperone dedicated to MHC-II molecules, CD74 is known to be a high-affinity receptor for Macrophage Migration Inhibitory (MIF).The aim of the present study was to define the roles of CD74 and MIF in the immunological surveillance escape process. To this direction, the leukemia cell lines HL-60, Raji, K562 as well as primary pre-B leukemic cells were used, whereas HeLa cells, lacking MHC-II molecules and Ii, were used as control cells. In order to clarify MIF's involvement in endosomal pathway, we examine along with secretion profile of MIF.
Materials and methods: Flow cytometry analysis was used to define expression of HLA-DR, CD74, HLA-DM, HLA-DO and MIF in HL-60, Raji, K562, HeLa and primary leukemic cells. Isolated mRNAs were submitted to RT-PCR experiments using CD74 specific primers. Regulation of CD74 and MIF expression by IFN-γ used as inducer was tested in K562 and HL-60 cell lines by flow cytometry analysis. The secretion of MIF in a free or exosomes-engaged form was evaluated by Elisa experiments.
Results: Flow cytometry analysis detected high levels of expression of MIF and CD74 at the membrane of all leukemic cells tested, whereas IFN-γ increased MIF expression and induced secretion of MIF in exosome-engaged forms.
Conclusions: The unexpected expression of MIF in leukemia cells and the detection of CD74 both intracellularly and membrane bound provides a novel mechanism for escape from immunological surveillance. CD74 seemed to possess a key-role in this pathway and react with MIF synergistically. Future studies determining the precise role of the CD74-MIF complex will allow understanding the involvement of MIF in the antigen presentation process.

P0622

Regulated release of ATP by Yac lymphoma cells

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Purpose/Objective: Extracellular ATP (eATP) concentrations are elevated in many tumors. By acting on purinergic receptors, eATP and its breakdown products exert multiple effects on tumors, including the transmission of survival signals, modulating the sensitivity to chemotherapeutic drugs, and positively or negatively influencing the anti-tumor immune response. Among other receptors, eATP acts on the P2X7 receptor, which is expressed by many cells of the immune system. While accumulation of eATP was thought to result mainly from necrotic tissue damage, awareness is increasing that many cell types actively secrete ATP in a regulated fashion. We used the Yac mouse T cell lymphoma to investigate whether lymphoma cells share this ability.
Materials and methods: Intracellular and extracellular ATP concentrations were measured in a luminometer using luciferase/luciferin. Dye uptake and Erk-phosphorylation were determined by flow cytometry.
Results: We first investigated whether TCR/CD3 signalling caused ATP release. Incubation of Yac cells with anti-CD3/CD28 antibodies decreased the concentration of intracellular ATP. Concomitant activation of P2X7 augmented this effect, and typically induced cells to release more than half of their intracellular ATP content. Surprisingly, we found that activation of P2X7 caused ATP release even in the absence of TCR signalling. Yac cells responded in multiple ways to activation of P2X7, including the shedding of cell surface CD62L and the externalisation of phosphatidylserine to the outer leaflet of the cell membrane – a characteristic of cells entering a preapoptotic stage. We also detected P2X7-dependant pore formation by monitoring the uptake of fluorescent dyes. We speculated that ATP might be released through the same pores that permit dye entry. However, preliminary pharmacological evidence suggests that ATP release and dye uptake occur by separate mechanisms. Stimulation by eATP strongly induced phosphorylation of the MAP kinases Erk1/2 in Yac cells, a pathway known to transmit anti-apoptotic survival signals to the cell.
Conclusions: In conclusion, our studies show that Yac lymphoma cells actively secrete substantial amounts of ATP into the extracellular environment in response to extracellular signals, including P2X7 activation. This released ATP may amplify P2X7 signalling or act on other purinoreceptors to modulate tumor growth and the anti-tumor immune response.

Poster session: Memory

P0623

Autoreactive-memory CD8+ T cells are unable to induce Type 1 diabetes

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Purpose/Objective: Autoimmune destruction of insulin-secreting beta-cells of the pancreas is responsible for the development of type 1 diabetes (T1D) and is mediated by self-reactive T cells that have escaped tolerance mechanisms. During the immune response to infectious agent, foreign antigen (Ag-specific naïve T cells undergo massive proliferation and differentiate into effector T cells that are responsible for the elimination of the pathogen. Following, Ag clearance, most of the effector T cells die while a few further (5−10%) differentiate into memory T (Tm) cells. Despite the implication of T cells in the development of T1D, few studies have investigated the role of memory T cells so far. Our goal is to evaluate the role of autoreactive Tm cells in the induction of autoimmune diabetes in a transgenic mouse model.
Materials and methods: To do this, we transferred different doses (5.10^4−3.10^6) of OT-I CD8+ Tm cells specific for ovalbulmin (OVA) in transgenic mice expressing OVA under the control of rat insulin (RIP-MOVA).
Results: Surprisingly, none of the different doses of Tm cells does not induce diabetes in recipient mice. In parallel, we observed that injection of 3.10^6 cells naïve OT-I allows the induction of diabetes. Following these results, we wanted to understand why Tm cells are unable to induce diabetes. First, we tested the localization of Tm cells transferred and we observed that these cells will migrate to the lymph pancreatic, therefore their location does not seem to be the problem. Secondly, we tested the activation of OT-I Tm cells compared to naïve OT-I cells *in vivo*. Interestingly, we observed that Tm cells are less activated than naïve T cells in pancreatic lymph nodes of RIP-mOVA, suggesting that Tm cells are more sensitive to the regulation by regulatory T cells than naïve T cells.
Conclusions: This comparative study of the functionality of two types of T cells in the context of diabetes could provide a better understanding of the mechanisms regulating the response of memory T cells.

P0624

CD4 memory T cells are resistant to the induction of tolerance *in vivo* despite poor survival following reactivation

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Purpose/Objective: Autoimmune diseases, such as rheumatoid arthritis, occur when an individual's immune system responds to their own organs, causing tissue damage and inflammation. A greater understanding of how to control the immune cells that can drive autoimmunity will provide the means to design targeted and rational therapeutics. Autoimmune diseases are, in the main, diagnosed following initiation of the disease. Any therapy that aims to tolerise autoreactive T cells must, therefore, target activated and memory cells rather than naïve T cells. Memory T cells can respond to low doses of antigen and costimulation, potentially allowing them to be activated by signals that successfully tolerise naïve T cells. While much is known about the means and mechanisms of tolerance in naïve CD4 T cells, there is little appreciation of whether and how memory CD4 T cells respond to tolerisation signals.
Materials and methods: We have used MHC class II tetramers and *in vivo* readouts of T cell effector functions to track and analyse the responses of endogenous CD4 T cells exposed to tolerisation signals *in vivo*.
Results: Antigen specific memory CD4 T cells proliferate in response to, and survive following, exposure to soluble peptide delivered in the absence of adjuvant. The subsequently generated memory cells express normal levels of chemokine and cytokine receptors such as CXCR5, CCR7 and CD127, suggesting that their migration and survival are unaltered by the tolerisation signal. Upon further activation *in vivo*, these memory cells divide normal but have a reduced ability to survive with increased caspase activation and reduced levels of Bcl2. Importantly, memory cells that have received a tolerisation signal are still able to provide an accelerated helper response for B cells resulting in the generation of antigen specific class switched antibody early in the response.
Conclusions: Memory cells respond to and re-enter the memory pool following exposure to a classical tolerisation signal. However, while these cells are not tolerant in terms of their proliferative or helper responses, their survival is limited following reactivation, suggesting a disconnect between T cell activation and survival.

P0625

CD69 and CD49b regulate the establishment and maintenance of T helper cell memory

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Purpose/Objective: Memory CD4 T cells play a key role in immunological memory. We have demonstrated that professional memory CD4 T cells reside and rest in the bone marrow (BM) adhering IL-7-expressing stromal niches. However, the cellular and molecular mechanisms of their establishment and maintenance in the BM remain unclear. We here show how effector CD4 T cells transmigrate to the BM and memory CD4 T cells adhere to their survival niches, mediated by CD69 and CD49b.
Materials and methods: To clarify the roles of CD69 and CD49b in the generation and maintenance of memory CD4 T cells in the BM, we transferred CD69 or CD49b-deficient or -blocked effector CD4 T cells into normal mice and evaluated their migaration ability to the BM, as well as the phenotypic or functional analyses of CD69 or CD49b-deficient CD4 T cells. Moreover, to understand the cellular mechanisms for establishment of memory cells in the BM, we performed the localization analyses of CD4 T cells in the effector or memory phase of an immune response.
Results: The loss or inhibition of CD69 or CD49b induced the defective migration of memory precursors into the BM. The inhibition of CD49b also reduced memory CD4 T cell numbers. Also, the localization analyses clarified the cell dynamics of memory precursors through CD69 and CD49b in the BM.
Conclusions: We here show that CD69 and CD49b regulate the establishment of memory CD4 T cells in the BM and also that CD49b supports the maintenance of memory cells. Furthermore, to mature into memory cells, memory precursors have to transmigrate into sinusoids and move onto survival niches for memory cells in the marrow of bones using CD69 and CD49b.

P0626

Demethylation at the IFNG gene promoter and CNS-1 correlates with cytokine memory for IFN-gamma in human CD4+ T lymphocytes

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Purpose/Objective: Cytokine memory for IFN-gamma by effector/memory Th1 cells plays a key role in both protective and pathological immune responses. To understand the epigenetic mechanism determining the ontogeny of effector/memory Th1 cells characterized by stable effector functions, we analyzed DNA methylation changes in the *IFNG* gene during Th1 differentiation of naïve human $CD4^+$ T cells, and we investigated how and at which stage these cells develope cytokine memory for IFN-gamma.
Materials and methods: *Ex vivo* $CD4^+$ and $CD8^+$ T cell subsets and NK cell subsets were isolated from healthy donors. For Th1 differentiation, naïve $CD4^+$ T cells were primed under Th1 conditions with allogenic monocyte-derived DCs or anti-CD3/CD28. *Ex vivo* or differented IFN-gamma-producing Th cells were isolated using cytokine capture assay and FACS. For *IFNG* reactivation, cells were stimulated with PMA/iono, fixed, permeablized and stained for intracellular IFN-gamma. DNA methylation analyses were performed using bisulfate-specific PCR sequencing, clone sequencing, and pyrosequencing. Cell cycle status was assessed by BrdU incorporation.
Results: We identified a T cell-specific methylation pattern at the promoter and CNS-1 of *IFNG* in *ex vivo* effector/memory Th1 cells. During Th1 differentiation, demethylation occurred at these two regions independently of cell proliferation and DNA synthesis. Early IFN-gamma-producers acquired 'permissive' levels of demethylation and developed into effector/memory cells undergoing progressive demethylation at these regions when induced by IL-12. Methylation levels of the promoter and CNS-1 in memory $CD4^+$ T cells of peripheral blood in RA patients correlated inversely with reduced frequencies of IFNG-gamma-producers, coincident with recruitment of effector/memory Th1 cellls to the site of inflammation.
Conclusions: TCR signaling and Th1 instructive signals induce dynamic demethylation of *IFNG* and the latter further stablizes *IFNG* expression during the transition from effector to effector/memory Th1 cells. Analyzing methylation status of *IFNG* may serve as a novel method for evaluating the distribution and/or re-distribution of effector/memory Th1 cells.

P0627

Differences in CD_4 and CD8 memory T-cell populations in Dutch children after vaccination with various pertussis vaccines

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Purpose/Objective: Whooping cough remains a worldwide problem despite vaccination. Since cellular immunity plays a role in long-term protection, we studied pertussis-specific T-cell responses. We compared T-cell memory responses in 4-years old children, who have been primed during infancy with either the whole cell pertussis (wP)- or the acellular pertussis (aP) vaccineand have been boosted with an aP vaccine at 4 years of age.
Materials and methods: PBMCs were isolated from 4-year old children and stimulated with the pertussis-vaccine antigens for 5 days. T-cells were phenotypically characterized by cell-staining for CFSE, CD4, CD3, CD45RA, CCR7, IFN-γ, TNF-α and dead cell discrimination. Cytokine responses were determined in culture supernatants by using fluorescent bead-based immunoassays.
Results: Three years after vaccination, at 4 years of age, T-cell cytokine responses were persistently high and T-cell proliferation ($CFSE^{dim}$) had increased in aP- compared to wP-vaccinated children. Pertussis-specific CD4 central memory T-cells ($CCR7^+$ $CD45RA^-$) were elevated just slightly, whereas clearly more effector memory cells ($CCR7^-CD45RA^-$) were induced in aP-vaccinated children compared to those vaccinated with wP. Upon stimulation with pertussis antigens CD8 T-cells reacted in a similar way as CD4 T-cells, except that about 15% exhausted T-cells were present in all samples. After a preschool aP booster vaccination, both T-cell effector memory CD4 and CD8 T-cells were enhanced in wP-vaccinated children, but not in aP vaccinated children.
Conclusions: Infant vaccinations with four high-dose aP vaccines result in high pertussis-specific CD4 and CD8 Tcell responses that persist in children until 4 years of age. After a preschool booster the already high T cell responses in aP vaccinated children did not increase in contrast to wP vaccinated children. Therefore, studies into changes in vaccine dosage and timing of aP pertussis (booster) vaccinations are needed.

P0628

Different populations of influenza antigen presenting cells in lungs regulate the proliferation and cytokine patterns of influenza specific memory T cells

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Purpose/Objective: To study phenotype, function and localization of influenza virus antigen presenting cells and virus specific memory T cells.
Materials and methods: Balb/c and C57BL/6 mice were infected intranasally with 10^4 $TCID_{50}$ of H_1N_1 PR8 influenza virus strain and 3 months later by a secondary infection via the same route with a 100 times higher dose of homologous influenza virus. At 3–6 months post last infection the mice were sacrificed and T cells were isolated from lymphoid organs and lungs. Influenza infected antigen presenting cells, APCs, were obtained from lymphoid organs and lungs of syngeneic mice infected intranasally for 36 h with 10^6 $TCID_{50}$ of H_1N_1 PR8 influenza virus. Viral RNA in organs was detected by RT-PCR analysis. Cells were purified by immunoaffinity with MiniMacs or with flow cytometry and responding T cells from influenza memory mice were co-cultured *in vitro* with different populations of influenza infected APCs.
Results: At 36 h post infection PCR analysis showed viral RNA in the lungs and in mediastinal lymph nodes but not in spleen or in inguinal lymph nodes. Intranasal challenge of memory mice with influenza virus rapidly induced IFN-γ containing memory T cells in lungs and spleen. Stimulation with influenza infected lymphoid APCs resulted in poor proliferation and cytokine production of the T cells *in vitro*. Dendritic cells and granulocytes in the lung down regulated proliferation of memory $CD4^+$ T helper cell *in vitro* while retaining a high production of pro-inflammatory IFN-γ and IL-17. The inhibited proliferation was enhanced by removal of $CD4^+$ $CD25^+$ regulatory T

cells from the responding $CD4^+$T cells. Furthermore influenza infected APCs in the lung promoted a strong $CD4^+$ T helper cell independent immune response of influenza specific $CD8^+$ memory T cells *in vitro*..
Conclusions: Intranasal infection of mice with influenza virus induces $CD4^+$ $CD25^+$ regulatory T cells and localizes different populations of influenza infected APCs in the lungs and its regional lymph nodes that can regulate the immune response of influenza specific memory T cells differently *in vitro*.

P0629
Effects of doxorubicin on bone marrow stroma and survival of long-lived plasma cells

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Purpose/Objective: Children treated with chemotherapy for leukemia loose antibodies they acquired from the childhood vaccination program, and the number of bone marrow plasma cells, crucial for maintenance of protective titers, is diminished. In addition, re-immunization is impaired at least up to a year after cessation of therapy. The bone marrow stroma is pivotal for homing and long-term survival of plasma cells, and consequently for vaccination memory. We hypothesize that cytotoxic drugs disrupt the plasma cell survival niche, due to stromal cell death and reduced production of cytokines, leading to impaired ability to support plasma cell homing and survival, and insufficient long-term antibody titers after vaccination.
Materials and methods: The commonly used chemotherapeutic agent Doxorubicin was used as model drug. The human derived stromal cell lines HS-5 and HS-27 were exposed to the same concentration of Doxorubicin as in plasma of pediatric ALL patients. Plasma cells were differentiated *in vitro* using peripheral blood from healthy donors.
Results: Semi-quantitative measurement showed significant uptake in the nuclei, and impairment of production of the known survival factors Interleukin-6, CXCL-12 and B-cell activating factor (BAFF) has been found. Preliminary data imply that stromal cell lines are able to increase plasma cell secretion of IgG, and further investigation will reveal weather the negative effects of Doxorubicin on stromal cells interfere with this ability.
Conclusions: We hope that our results will add important clues to how cytotoxic treatment can have long-term effects on immunity, and consequently why vaccination is not always possible after leukemia treatment.

P0630
Human memory B cell response and its age-dependence after tick-borne encephalitis virus vaccination

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Purpose/Objective: The ability of the immune system to respond to antigenic stimulation decreases with advanced age which leads to reduced protective effects of vaccination and increased morbidity due to infectious diseases. To date, there is little information about the frequency and functional integrity of memory B cells generated during old age.

We have conducted a prospective analysis of the quantities and functionalities of antigen-specific B cell responses and its association with the functional helper $CD4^+$T cell responses. The ability of naïve B cells from old and young humans to establish functional memory was examined following primary and booster vaccination with an inactivated-virus vaccine against tick-borne encephalitis.
Materials and methods: For this purpose, we quantified TBEV-specific IgG, neutralizing antibody titers and TBEV-specific memory B cells using an ELISA-based limiting dilution analysis in 12 young (20−31 years) and 21 older (60−80 years) healthy individuals.
Results: Our data show that the number of antigen-specific memory B cells generated during primary vaccination was ~3-fold lower in old than in young individuals. The maintenance and booster responsiveness of these memory B cells was not compromised, as evidenced by similar increases in specific memory B cell frequencies upon revaccination in old and young vaccinees. In contrast, the Ab response mediated per memory B cell after revaccination was dramatically diminished in the elderly. Also, antigen-specific IL-2-positive $CD4^+$T cell responses were strongly reduced in the elderly and displayed an excellent correlation with Ab titers.
Conclusions: The data suggest that the dramatically lower antibody response in the elderly could only partially be accounted for by the reduced B cell numbers and was strongly correlated with profound functional defects in CD4 help.

P0631
Identification of resting bone marrow memory T cells in humans

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Purpose/Objective: Memory T cells play a critical role in immunological memory responses, in that they mediate more effective protection during pathogen rechallenge. We have previously shown that murine memory $CD4^+$ T cells characterized by expression of Ly-6C preferentially reside and rest in the bone marrow (BM). Here we aimed to address whether human resting memory T cells also reside and rest in the BM, and we dissect the functional properties of memory T cells isolated from the BM and the periphery.
Materials and methods: Mononuclear cells were isolated from paired blood and BM samples from healthy individuals. Phenotypic analysis and cytokine profile of distinct memory T-cell subets were assessed by flow cytometry. Proliferation and cell cycle status were analyzed using Ki-67 and PI staining, respectively. Signature molecules for resting memory T cells and signaling pathways required for the maintenance of bone marrow memory T cells will be identified by transcriptome analysis. Antigen specificity will be assessed by activation of memory T-cell subsets with a panel of antigens of interest. Mobilization of antigen-specific memory T cells to the periphery will be performed by systemic re-immunisation of healthy donors with tetanus toxoid.
Results: Distinct CD69-expressing $CD8^+$ $CD45RA^-$ and $CD4^+$ $CD45RA^-$ T cells can be detected in the bone marrow but not in the peripheral blood. CCR7 expression was decreased on bone marrow $CD69^+$ $CD4^+$ $CD45RA^-$ and $CD69^+$ $CD8^+$ $CD45RA^-$ T-cells. Ki-67 staining indicated that T cells from the bone marrow proliferated less than those from the peripheral blood.
Conclusions: In steady state, human bone marrow consists of CD69-expressing $CD8^+$ $CD45RA^-$ and $CD4^+$ $CD45RA^-$ T cells. Compared to peripheral memory-phenotype T cells, bone marrow memory-phenotype T cells reduce their homing capacity to the secondary lymphoid organs by downregulating CCR7 expression, and rest in terms of proliferation. Future work will identify signature molecules for resting memory T cells and their functional properties.

P0634

The role of CMV in development of the human T cell memory repertoire and the influence of co-infection with EBV

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Purpose/Objective: 70% of people acquire cytomegalovirus (CMV)-infection by adulthood. Usually asymptomatic it can cause severe complications in immune suppressed. The virus establishes latency with episodes of reactivation. In healthy donors the immune system controls this, but a large proportion of the T cell compartment is dedicated to CMV and virus specific T cells accumulate leading to impaired control of other infections. The aim of this study was to investigate changes CMV-infection induces in the T cell repertoire of young healthy donors, how stable the immune response against this virus is over time and how it is influenced by co-infection with other viruses e.g. Epstein-Barr virus (EBV). For this purpose we started a large prospective study of a student cohort.

Materials and methods: Blood samples were taken at time of entry into medical school and yearly thereafter, Plasma and PBMCs were isolated and cryostored. Serostatus for CMV and EBV was determined by ELISA or VCA-test. Using multicolour flow cytometry the overall T-cell repertoire, frequencies and phenotype of virus specific T-cells were analysed. EBV viral load in PBMC was determined by qPCR.

Results: Four donor groups were identified: CMV^-/EBV^- (17%), CMV-/EBV+ (44%), CMV+/EBV^- (10%) and CMV+/EBV+ (29%). The group with dual infection showed a marked reduction in the CD4:CD8 T cell ratio. Comparing the overall T cell repertoire no differences were seen between virus naïve donors and those infected with EBV only. Single infection with CMV caused profound changes in both CD4 and CD8 T cell repertoire. A large reduction in the expression of costimulatory molecules CD27 and CD28 * 15% and 5% on CD4+, 25% and 17% on CD8+ T cells and homing marker CD62L (16% for CD4+ and 18% for CD8+ T cells) was observed, also a marked increase in the frequency of differentiated CD57+ T cells. Concerning memory T cell subsests CMV infection induced a significant reduction in the frequency of naïve T cells and a marked increase of effector memory cells. These changes were less pronounced in CMV+/EBV+ individuals. Frequencies of EBV-specific T cells were lower in those who are co-infected with CMV.

Conclusions: Already at a young age CMV infection shapes the immune repertoire dramatically. Changes seem to be more pronounced in individuals that were infected with CMV only compared to those who were co-infected with EBV.

Poster Session: NKT and γ T Cells

P0636
$CD56^+$ $CD3^+$ T cells in relation to infection in normal subjects and kidney transplant patients

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Purpose/Objective: $CD3^+$ $CD56^+$ cells are a small subset of T cells of unknown function but with cytotoxic capacity following activation *in vitro*. In preliminary work we found that levels were higher in normal subjects positive for cytomegalovirus (CMV) than in CMV- subjects. Kidney transplant patients are particularly prone to reactivation of CMV infection which as well as causing acute disease may play a role in triggering the rejection process. Aims; This work aims to study $CD3^+$ $CD56^+$ T cells and their response to CMV and other pathogens, and their possible role in triggering the immune system toward the transplanted organ.
Materials and methods: Phenotypic studies of several markers of $CD56^+$ $CD3^+$ in normal CMV^+ and CMV- subjects were done to investigate the lineage of these cells by FACS analysis and whether they are increased in CMV infection. Any differences in numbers, phenotype or function in transplant patients with or without CMV infection were analysed.
Results: as have been found in our preliminary study, $CD3^+$ $CD56^+$ are higher in CMV^+ in both healthy and patients PBMC than CMV-. Moreover, CD62L, CD161 and CD28 are higher in CMV- healthy and patients PBMC than CMV^+ healthy and patientsPBMC. CD161 is known to be an inhibitory function in NK cells which may also serve the same role in NKT cells. CD62L is a memory and effector marker found in naïve cells, and their low expression on NKT indicates previous stimulation of these cells. On other hand, the costimulatory molecule CD28 is less in CMV^+ than CMV- which could be lost after these cells being activated.
Conclusions: Conclusion and results are written on the Results Box

P0638
Column-free isolation of untouched human gamma-delta T cells from PBMC in 45 min

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Purpose/Objective: T cells expressing the γδ T cell receptor (γδ TCR) make up a minor subset of human circulating T cells (1–10%). γδ T cells are distinct from αβ T cells in that they exhibit limited combinatorial diversity of the TCR and recognize non-peptide antigens independent of HLA molecules. γδ T cells exert innate effector functions including rapid release of cytokines and killing of target cells without prior antigen exposure. Adaptive immune responses such as memory functions have also been attributed to γδ T cells. Typically, elaborate purification protocols such as FACS-based cell sorting or expansion in culture are needed to obtain enough γδ T cells for subsequent studies. Here, we describe an immunomagnetic, column-free negative selection method (EasySep™) to isolate untouched γδ T cells from fresh or previously frozen peripheral blood mononuclear cells (PBMC).
Materials and methods: Briefly, non-γδ T cells are labeled and cross-linked to dextran-coated magnetic particles using bispecific tetrameric antibody complexes. γδ T cells were then separated from unwanted labeled cells by placing the tube in an EasySep™ magnet and pouring off unlabeled cells into a new tube. The enrichment procedure may also be performed using the automated cell separator, RoboSep™. Functionality of the enriched cells was tested by evaluation of cytokine production in response to aminobisphosphonate (zoledronate) pretreated monocytes.
Results: Starting with 3 ± 2% $CD3^+$ γδTCR^+ T cells in PBMC, purities of 94 ± 3% (n = 20) were achieved using EasySep™. Similar purities were observed using RoboSep™. The isolated γδ T cells produced interferon-gamma (IFN-γ) when activated with zoledronate-pretreated monocytes.
Conclusions: γδ T cells have potential therapeutic applications in cancer and infectious diseases. This rapid method for the isolation of γδ T cells will assist in the study of γδ T cell biology and the development of γδ T cell-based immunotherapies.

P0639
Direct Identification of Rat iNKT Cells Reveals Remarkable Similarities to Human iNKT Cells and a profound deficiency in LEW Rats

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Purpose/Objective: Human iNKT cells differ from that of mice in terms of frequency (about 0.5% of intrahepatic T cells compared to 50% in C57BL/6 mice), surface phenotype (some CD8aa positive in humans but not in mouse) and *in vitro* expandability (very easy in humans but not in mouse). iNKT cells of rats, which serve as model for many human diseases, have not yet been directly identified, although genetic as well as functional studies (CD1d dependent a-GalCer response) have indicated their existence. Aim of this study is phenotypical and functional characterization of rat iNKT cells.
Materials and methods: First generation of rat CD1d dimers which allow direct rat iNKT cell identification. Identification of *AV14AJ18* rearrangements of different *AV14* genes (type I versus Type II) by sequencing of RT-PCR products in different organs. Phenotypical rat iNKT cells by immuno-flow cytometry including intracellular PLZF staining and and functional characterization by cytokine assays.
Results: RatCD1d dimers were found to be mandatory for direct identification of iNKT cells in the rat and allowed first time identification of these cells. Rat iNKT cells expressed PLZF and were CD4 positive, double negative and to a very low frequency CD8aa positive. Cells were found to produce IL-4 and IFNg by intracellular staining, ELISPOT and ELISA. The surface phenotype and the very low frequency of iNKT cells(1.05% ±0.52 (SD) of all intrahepatic T cells) found in F344 rats resembled features of human iNKT cells. F344 rat iNKT cells resembled human iNKT cells also in their robust *in vitro* expansion. In contrast, in LEW inbred rats, which are often used as model for organ-specific autoimmune diseases, iNKT cells were near or below the detection limit. Interestingly, the usage of members of the rat *AV14* gene family differed between F344 and LEW inbred rats. Differences in the CD1d binding properties of several members of this gene family are currently analyzed *in vitro*.
Conclusions: The resemblance of F344 inbred rat iNKT cells to human iNKT cells and the availability of the iNKT cell-deficient LEW rat strain makes the rat a promising animal model for the study of iNKT cell-based therapies and of iNKT cell biology.

P0640

Evolution of Vg9Vd2 T cells

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Purpose/Objective: The hallmark of Vgamma9Vdelta2 (Vγ9Vδ2) T cells is expression of Vγ9JP and Vδ2 containing TCR chain with reactivity for pyrophosphate containing metabolites (phosphoantigens) of host and microbial isoprenoid synthesis such as IPP and HMBPP. Rodents lack Vγ9 and Vδ2 like genes and so far it is generally perceived that phosphoantigen-recognizing Vγ9Vδ2 T cells are restricted to higher primates. Aim of this study is to test for existence of Vγ9Vδ2 T cells and phosphoantigen-reactivity in other mammalian taxa and to use this knowledge for analysis of molecular basis of antigen recognition by Vγ9Vδ2 T cells and their phosphoantigen-mediated activation.
Materials and methods: RNA extracted from PBMCs of alpaca and horse lymphocytes were used for cDNA synthesis, which was later used for Reverse transcriptase PCR. BovineMacrophage Cell line (BoMac) was used as the source of genomic DNA. Genomic DNA and cDNA was used for amplification of Vγ9 and Vδ2 like genes and PCR products were cloned into TA vector and analyzed by sequencing.
Results: Analysis of public data bases revealed fragments of Vγ9 and Vδ2 like genes in all eutherian subgroups (Afrotheria, Xenarthra, Euarchontoglires and Laurasiatheria) suggesting common emergence of Vγ9 and Vδ2 genes and placental mammals. So far we identified full length Vγ9 and Vδ2 like sequences including RSS in six species namely, 1.Horse 2.Cow 3.Alpaca 4.Sloth 5.Bottlenose dolphin 6.Grey mouse lemur. The first three were further analyzed by (RT)-PCR and sequencing. Horse and cow genome showed Ig domain disrupting mutations and stop codons, respectively. But RT-PCR of alpaca (*vicugna pacos*) blood mononuclear cells revealed Vγ9JP and Vδ2 comprising rearrangements spliced to the respective C domains. This suggests the capacity to express Vγ9Vδ2 TCR. Analysis of alpaca blood cells for phosphoantigen-reactivity and attempts to clone, express and analyze function of the presumed alpaca Vγ9Vδ2 TCRs are in progress as well as analysis of co-evolution with members of the buytrophilin-family.
Conclusions: Vγ9 and Vδ2 TCR emerged with eutherian evolution. Analysis of in frame Vγ9JP and Vδ2 containing TCR-chains suggests existence of functional Vγ9Vδ2 T cells in alpaca – a new world camelid – and offers the opportunity to study function and evolution of this cell population.

P0641

HLA class I molecules in tumor B cells: stress antigens for Vdelta3 gamma-delta TCR?

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Purpose/Objective: Non-Vδ2 $\gamma\delta$ T cells represent the vast majority of $\gamma\delta$ T cells in epitheliums and tissues. These subsets show high TCR diversity, and most of the antigenic ligands of non-Vδ2 $\gamma\delta$ TCR remained unidentified. Our previous studies evidenced an expansion of non-Vδ2 $\gamma\delta$ T cells in Cytomegalovirus (HCMV) infected individuals. $\gamma\delta$ T cell clones isolated from these patients show dual reactivity against tumor cell lines and HCMV infected cells suggesting recognition of stress self antigens. The aim of this study was to identify antigens recognized by Vδ3 $\gamma\delta$ T cells.
Materials and methods: In order to determine the cell targets expressing antigenic ligands of Vδ3 $\gamma\delta$ TCR, we generated Vγ9Vδ3 TCR transductants derived from an HCMV-reactive $\gamma\delta$ T cell clone. Target cell lines were co-cultured with Vγ9Vδ3 TCR transductants or the original clone and TCR activation was measured by flow cytometry using CD69 / CD107a markers. Highest activating cells were used to immunize mice and hybridomas supernatants were screened for blocking Vγ9Vδ3 TCR activation. Analyses were performed using selected antibodies to identify antigens recognized by the TCR.
Results: Among 50 tumor cell lines tested, only three $CD20^+$ /$CD38^+$ tumor B cell lines specifically activated Vγ9Vδ3 TCR transductants. Several mAbs (LSM-01 to 06) abrogating the anti-B cell reactivity of Vγ9Vδ3 TCR transductants and original clone were generated. They ubiquitously stained different cell lines except HLA class I-deficient ones. Recognition of HLA-I by LSM mAbs was confirmed by immunoprecipitation experiments, and their reactivity towards HLA class I alleles established using HLA-typing Luminex technology. Alignments of HLA sequences revealed a shared epitope for all LSM mAbs in the α-2 domain of HLA-I proteins. The silencing of β-2-microglobulin in B cell targets abrogated Vγ9Vδ3 TCR reactivity. Interestingly, original clone expressed HLA-inhibitory receptors suggesting a regulation of TCR signal in physiologic context. Blocking these receptors dramatically increased the Vγ9Vδ3 $\gamma\delta$ T clone reactivity against B cell targets.
Conclusions: Taken as a whole, our results identify HLA-I molecules as potential stress antigens recognized by a Vγ9Vδ3 TCR. Dual reactive clones and the TCR they express constitute interesting tools to identify stress antigens of $\gamma\delta$ TCRs, which is critical to consider $\gamma\delta$ T cell activation for therapy purposes.

P0642

IL-17 production by gamma delta T cells contributes to the clearance of persistent bacterial renal infection

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Purpose/Objective: The innate immune system and particularly polymorphonuclear neutrophilic granulocytes (PMNs) are the first responders to, and are responsible for, the clearance of bacterial infections. IL-17 has been established as one of the key cytokines in pathways of PMN recruitment and activation. In addition to the well-known Th17 cells, another major producer of IL-17 are the innate-like leukocyte gamma delta ($\gamma\delta$) T cells. Here we investigate the role played by $\gamma\delta$ T cells in the clearance of renal bacterial infections in pyelonephritis.
Materials and methods: The clinical uropathogenic *E. coli* isolate 536 (UPEC 536) was instilled into the bladder of anaesthetised C57Bl/6 mice using a catheter. Mice were infected again 3 h later to induce an ascending renal infection. Kidneys were excised and digested with DNAse/Collagenase and the inflammatory infiltrate analysed by flow cytometry. The level of kidney infection was determined by plating dilutions of the renal digest onto CPS ID plates and counting colonies.
Results: Kidney infection with *E. coli* was maximal 3 h after the second infection, with the numbers of *E. coli* present dropping 1000-fold over the following 72 h. Infiltration of the renal tissue by PMNs peaked at day 2 post-infection and decreased sharply as the bacteria were cleared. However, PMN numbers rose again if the bacteria were not cleared and a chronic infection was established. Notably although $\gamma\delta$ T cells were extremely rare in the normal kidney, their numbers increased significantly from fourfold at 72 h post-infection up to 12-fold at day 7 post-infection. Furthermore the numbers of $\gamma\delta$ T cells remained slightly elevated in mice that had cleared the infection as compared to healthy mice. When the cytokine profile of the $\gamma\delta$ T cells was investigated they produced IL-17 rather than IFNγ (27.3% versus 2.4% respectively). Additionally $\gamma\delta$ T cells predominately produced IL-

17A alone (17.6%) rather than IL-17A and IL-17F (3.9%) or IL-17F alone (4.4%).
Conclusions: Bacterial renal infection or pyelonephritis induces the infiltration of $\gamma\delta$ T cells into the renal parenchyma where they express IL-17A. The expression of IL-17 by $\gamma\delta$ T cells later in renal infection may be responsible for amplification of the innate immune response, enabling optimal recruitment and activation of PMNs and clearance of persistent renal infections.

P0644
Implication of gamma delta T cells in the immune response against murine CMV

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Purpose/Objective: We previously showed that $\gamma\delta$ T cells participate to CMV riddance in humans: 1- CMV-infection is associated with activation and expansion of circulating $\gamma\delta$ T cells, 2- CMV-induced $\gamma\delta$ T cells control CMV multiplication through Interferon γ release *in vitro* and 3- $\gamma\delta$ T cell expansion is concomitant to the resolution of viremia in immune-compromised individuals. Further understanding of $\gamma\delta$ T cell-mediated control of CMV requires the development of a reliable animal model. The aim of the present study was then to determine whether murine $\gamma\delta$ T cells are involved in the control of mouse CMV (MCMV).
Materials and methods: C57BL/6 mice deficient for $\gamma\delta$ and/or $\alpha\beta$ T cells were infected with MCMV in order to compare their sensitivity towards MCMV as well as the viral load in different organs.
Results: We showed that whichever subpopulation is sufficient to confer protection of mice against MCMV. DNA copy numbers of MCMV increased substantially with time in all organs tested from CD3$\varepsilon^{-/-}$ infected mice until they died. At that time however, viral loads were much lower in TCR$\alpha^{-/-}$ mice when compared to CD3$\varepsilon^{-/-}$ mice. Finally, reconstitution of $\gamma\delta$ T-cell development in CD3$\varepsilon^{-/-}$ mice using bone marrows from TCR$\alpha^{-/-}$ mice confers protection against MCMV.
Conclusions: Altogether these results indicate that murine $\gamma\delta$ T cells are involved in the control of MCMV uncovering an interesting model to dissect the anti-viral functions of this enigmatic unconventional T cells. They also suggest that $\gamma\delta$ T cells could compensate for the absence of $\alpha\beta$ T cells in the fight against CMV, a property that could be of particular relevance in immune-suppressive contexts where $\alpha\beta$ T cells are more specifically compromised.

P0645
iNKT cell crosstalk with dendritic cells in the splenic marginal zone drives CCL17 production in a STAT6-dependent manner

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Purpose/Objective: CD8$^+$ T cell activation is a tightly regulated process that requires a range of signals provided by mature dendritic cells (DCs). For DCs to be able to provide these stimulatory signals, they first need to be activated in a process called DC licensing, which can be performed by invariant NKT (iNKT) cells. Chemokines and their receptors seem to be important mediators of the cellular interactions required for cognate help. We could previously show, that induction of the chemokine CCL17 is a part of NKT cell-mediated DC licensing, but how iNKT cells mediate this event *in situ* remains elusive.
Materials and methods: CCL17 expression was analysed by flow cytometry and qRT-PCR. iNKT cell visualization was performed by a confocal imaging approach using CD1d tetramers to identify the location of endogenous iNKT cells *in situ*.
Results: We now show that DC-derived CCL17 is induced by NKT cell-derived IL-4 in a STAT6-dependent manner. Using a new method to unambiguously localize the endogenous splenic iNKT cell population, we demonstrate that upon administration of microbial and synthetic glycolipid antigens, iNKT cells rapidly accumulate in the splenic marginal zone where they produce IL-4 in a highly compartmentalized fashion. iNKT cell activation requires marginal zone DCs and acts reciprocally to upregulate CCR7 and mobilize this antigen-presenting subset to the splenic T cell zone for interaction with CD8$^+$ T cells.
Conclusions: These new insights into intrasplenic immune cross-talk demonstrate a critical role for both Type 2 cytokine signaling and cognate interactions in the iNKT cell-dependent DC licensing program.

P0646
Interleukin 17 responses induced in mice by chemical allergens: a functional role for ydeltaT cells

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Purpose/Objective: The interleukin (IL)-17 cytokine family, expressed by T helper 17 cells, plays pivotal roles in adaptive immune responses. They have been implicated in autoimmune and allergic diseases as well having roles in bacterial and fungal clearance. Importantly, several innate sources of these cytokines have also been described, including $\gamma\delta$ T cells. Interestingly, IL-17 producing $\gamma\delta$ T cells have been shown to be important in the development of adaptive Th17 responses during experimental autoimmune encephalomyelitis. We have questioned whether IL-17 influences the development of sensitisation to low molecular weight chemical allergens.
Materials and methods: BALB/c strain mice were exposed topically to the contact allergen 2,4-dinitrochlorobenzene (DNCB), the respiratory allergen trimellitic anhydride (TMA), or to vehicle alone. At selected time points single cell suspensions of draining lymph nodes were cultured and analysed for cytokine secretion (ELISA) or for mRNA following enrichment/depletion using magnetic beads.
Results: A single exposure to either allergen resulted in transient up-regulation of IL-17 from $\gamma\delta$ T cells in the draining lymph node. Maximal levels of secretion were observed at 6 and 48 h following exposure to DNCB and TMA, respectively. After repeated exposure under conditions where DNCB and TMA stimulate polarised Th1 and Th2 cytokine phenotypes, respectively, IL-17 production by DNCB-activated cells was shown by complement depletion to reside in the CD4$^+$ population only. In subsequent experiments, responses were explored in $\gamma\delta$ T cell KO mice and C57BL6 wild type (WT) controls. A similar pattern of IL-17 production to that provoked in BALB/c strain mice was seen in the WT mice following prolonged exposure to DNCB. However, in the $\gamma\delta$ T cell KO mice the adaptive Th17 response was completely abrogated.
Conclusions: These data suggest strongly that the lack of IL-17 production by $\gamma\delta$ T cells during the acute (innate) response affects the subsequent adaptive Th17 response to chemical contact allergens.

P0647

Interleukin-7 selectively promotes expansion and functional properties of mouse and human IL-17-producing gamma/delta T cells

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Purpose/Objective: IL-17-producing CD27$^-$ $\gamma\delta$ cells ($\gamma\delta^{27-}$ cells) are widely viewed as innate immune cells making critical contributions to host protection and autoimmunity. However, factors that promote them over interferon-γ-producing $\gamma\delta^{27+}$ cells are poorly elucidated. Moreover, although human IL-17-producing $\gamma\delta$ cells are commonly implicated in inflammation, the cells themselves have proved difficult to isolate and characterise.
Materials and methods: Therefore, in our study, we investigated *in vitro* and *in vivo* the role of cytokines on the proliferation and the functional properties of mouse and human IL-17-producing $\gamma\delta$ cells.
Results: Our data show that murine CD44^{high} $\gamma\delta^{27-}$ cells and thymocytes are rapidly and substantially expanded by IL-7 *in vitro* and *in vivo*, notably in the context of imiquimod-induced inflammation. This is not the case for IFN-γ-producing ($\gamma\delta27^+$) cells. This is in large part attributable to the capacity of IL-7 to activate STAT3 selectively in $\gamma\delta^{27-}$ cells. IL-7 also permits IL-17-producing $\gamma\delta$ cells to respond to TCR agonists rather than to be killed by them, thus re-emphasizing the cells' adaptive as well as innate potentials. This cytokine facilitates notably strong responses, such as cytotoxicity, of $\gamma\delta^{27-}$ cells to TCR stimulation. Likewise, IL-7 and TCR agonists jointly drive the substantive expansion of IL-17 producing human $\gamma\delta$ cells.
Conclusions: Hence, IL-7 is a profound, conserved, and selective regulator of IL-17-producing $\gamma\delta$ cells, with both biological and clinical implications.

P0648

NKT cells aggravate the development of abdominal aortic aneurysms

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Purpose/Objective: An abdominal aortic aneurysm (AAA) is a dilatation of the abdominal aorta and is mostly undiscovered until it ruptures leading to serious complications and mostly to death. Development of AAA is associated with an accumulation of inflammatory cells in the lesions such as NKT cells. The exact contribution of these cells in AAA formation remains unclear. The goal of this study was to investigate the role of NKT cells in angiotensin II (AngII) induced AAA formation.
Materials and methods: To investigate the influence of AngII on NKT cell activation, an NKT cell hybridoma and splenocytes from LDLr$^{-/-}$ mice were incubated with AngII in combination with α-GalCer. Additionally, to determine the role of NKT cells in AAA formation, LDLr$^{-/-}$ and LDLr$^{-/-}$CD1d$^{-/-}$ mice were fed a Western-type diet prior to infusion with AngII.
Results: *In vitro*, AngII amplified the α-GalCer induced activation of NKT cells observed by increased production of IL-2 by the NKT cell hybridoma and increased IFN-γ production by splenocytes. Additionally, *in vivo*, AngII infusion induced an increased expression of the activation marker CD25 on NKT cells. During the *in vivo* aneurysm experiment, 5 out of 12 LDLr$^{-/-}$ mice died due to rupture of the aorta while in the LDLr$^{-/-}$CD1d$^{-/-}$ group 0 out of 11 mice died. A clear significant difference in severity of AAA was observed in the surviving mice. In seven out of 11 LDLr$^{-/-}$CD1d$^{-/-}$ mice no AAA lesions were found compared with only one out of 12 LDLr$^{-/-}$ mice.
Conclusions: These data show that NKT cells aggravate the development of AAA and induce aortic ruptures in an AngII-based AAA model possibly due to a direct effect of AngII on NKT cells. These results provide new opportunities to intervene in the development of aneurysms.

P0649

NKT cells play a role in an experimental model of adenine-induced renal injury

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Purpose/Objective: Diseases that affect the renal tubulointerstitial compartment are numerous and varied. The pathogenesis of kidney diseases involves the participation of various elements of the immune response. NKT cells constitute a distinct population of lymphocytes characterized by reactivity to glycolipids presented by CD1d molecules. The aim of our study was to analyze the role of NKT cells in an experimental model of adenine-induced tubulointerstitial nephritis.
Materials and methods: We used wild type C57BL/6j mice and Jα18 KO and CD1d KO mice (8–10 weeks old). A diet containing 0.25% adenine was given *ad libitum* to the animals for 10 days. Control animals were fed a standard diet. A second group of wild type (WT) animals was injected once with agonists of NKT cells, αGalCer or Sulfatide, and the adenine dietwas started at the same day. All groups were euthanized on the 10th day after adenine diet feeding. Blood and kidney samples were collected for analysis of renal function, gene expression, histology and immunohistochemistry. Data were presented as mean ± SD and ANOVA was used to compare groups.
Results: Jα18 KO and CD1d KO mice which were fed adenine diet had a significant increase in gene expression of KIM-1 and TNF-α in renal tissue compared to WT adenine fed mice. There was also a significant increase in renal interstitial fibrosis in Jα18 KO mice of the adenine group compared to WT mice of the same group. αGalCer and Sulfatide administration reduced serum creatinine levels in adenine fed WT mice. Similarly, a reduction of IL-6 and TNF-α gene expression was also observed after αGalCer and Sulfatide administration. Most importantly, there was a significant decrease of interstitial fibrosis and staining for FSP-1 and α-SMA following αGalCer and Sulfatide injection and adenine feeding compared to WT mice of adenine group.
Conclusions: Taken together, these data indicate that NKT cells participate in the process of adenine-induced renal injury by attenuating it. The absence of NKT cells leads to a deterioration of renal function and its activation is capable of improving kidney function in adenine-fed WT mice.

P0650

NOD mice contain an elevated frequency of iNKT17 cells that exacerbate diabetes

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Purpose/Objective: Invariant natural killer T (iNKT) cells are a distinct lineage of innate-like T lymphocytes and converging studies in mouse models have demonstrated the protective role of iNKT cells in

the development of type 1 diabetes. Recently, a new subset of iNKT cells, producing high levels of the pro-inflammatory cytokine IL-17, has been identified (iNKT17 cells). Since this cytokine has been implicated in several autoimmune diseases, we have analyzed iNKT17 cell frequency, absolute number and phenotypes in the pancreas and lymphoid organs in non-obese diabetic (NOD) mice.
Materials and methods: iNKT17 cells frequency in lymph nodes and in the pancreas was determinated by flow cytometry mice. Local activation of this cells was determined by qPCR. The role of iNKT17 cells in the development of diabetes was investigated using transfer experiments.
Results: NOD mice exhibit a higher frequency and absolute number of iNKT17 cells in the lymphoid organs as compared to C57BL/6 mice. iNKT17 cells infiltrate the pancreas of NOD mice where they express IL-17 mRNA. Contrary to the protective role of $CD4^+$ iNKT cells, $CD4^-$ iNKT cells containing iNKT17 cells enhance the incidence of diabetes. Treatment with blocking an anti-IL-17 antibody prevents the exacerbation of the disease.
Conclusions: This study reveals that different iNKT cell subsets play distinct roles in the regulation of type 1 diabetes. iNKT17 cells that are abundant in NOD mice, exacerbate diabetes development. Further investigations are underway to analyze iNKT17 cell activation in the pancreas.

P0651
Porcine gammadelta T lymphocytes differ in level of TCRgamma-delta expression

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Purpose/Objective: Our studies showed that natural expression of T cell receptor (TCR) $\gamma\delta$ on fresh porcine T lymphocytes occurs in two densities: low and high. These two subsets have different distribution of CD2/CD8 subsets of porcine $\gamma\delta$ T cells. It is known that different CD2/CD8 $\gamma\delta$ T cell subsets are differentially located in various tissues and they likely have different functional characteristic. However, there is no report showing whether the CD2/CD8 subsets represent separate and independent lineages or if they represent subsequently developing subsets.
Materials and methods: Flow cytometry and cell sorting; cell cultures and stimulation *in vitro*; PCR amplification and CDR3 spectratyping.
Results: Natural expression of T cell receptor (TCR) $\gamma\delta$ on fresh porcine T lymphocytes occurs in two densities: low and high. These two subsets are differentially distributed among CD2/CD8 $\gamma\delta$ T cells: while $TCRgd^{lo}$ T cells are preferentially $CD2^+$ $CD8^+$, $TCR\gamma\delta^{hi}$ T cells are mostly $CD2^{\tilde{N}}CD8^{\tilde{N}}$. Sorting and cultivation experiments revealed that $TCR\gamma\delta^{hi}$ T cells can generate $TCR\gamma\delta^{lo}$ T cells in cultures but never oppositely. Most intense down-regulation of $TCR\gamma\delta$ can be observed in $CD2^{\tilde{N}}CD8^{\tilde{N}}$ subset. Moreover, stimulation by PMA or IL-2 can enhance the down-regulation of $TCR\gamma\delta$. However, while IL-2 has clearly highest impact on $CD2^{\tilde{N}}CD8^{\tilde{N}}$ $\gamma\delta$ T cells, PMA preferentially influence $CD2^+$ $CD8^+$ subset. It has been also shown that $CD2^{\tilde{N}}CD8^{\tilde{N}}$ $\gamma\delta$ T cells are unable to generate other CD2/CD8 subsets while $CD2^+$ $CD8^{\tilde{N}}$ and $CD2^+$ $CD8^+$ can acquire or lost CD8 molecule respectively. In accordance, there is significantly more cycling $CD2^+$ $CD8^{\tilde{N}}$ and $CD2^+$ $CD8^+$ than $CD2^{\tilde{N}}CD8^{\tilde{N}}$ $\gamma\delta$ T cells after IL-2 *in vitro* stimulation. Interestingly, although IL-2 clearly stimulates all three CD2/CD8 subpopulations, while $CD2^+$ $CD8^{\tilde{N}}$ and $CD2^+$ $CD8^+$ almost do not express CD25, $CD2^{\tilde{N}}CD8^{\tilde{N}}$ $\gamma\delta$ T cells respond by significant increase in CD25 expression. These results collectively indicate that CD2 expression most probably determine two lineages of $\gamma\delta$ T cells that differ in many functional, phenotype and behavioral aspect: (1) $CD2^{\tilde{N}}CD8^{\tilde{N}}$ and (2) $CD2^+$ $CD8^{\tilde{N}}$ and $CD2^+$ $CD8^+$ $\gamma\delta$ T cells.
Conclusions: This work was supported by grants P502/12/0110 and P502/10/0038 from Czech Science Foundation, GA UK 151-43-251119 and MSMT ME09089.

P0652
Regulatory network of T regulatory cells (Tregs) and NKT cells in environmental diseases

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Purpose/Objective: Objectives: The occurrence of environmental diseases has been increasing more than 30% for the last few decades and these illnesses are an onerous burden both to the individual and to the public health. These diseases include all disturbances caused by environmental, as well as behavioral factors. Environmental factors represent the group of changeable outdoor and indoor environments, while behavioral influences involve the augmentation in immunizations as a result of frequent infections, non regular use of antibiotics, diminished physical activities and decreased outdoor staying. The augmentation of NKT cells is associated with antitumor and allergic immune response, chronic inflammatory conditions, viral infection and autoimmune diseases. Moderation of immune response by Tregs is characterized with specific T-cell proliferation, following with decreased secretion of T-helper 1 and T-helper 2-type cytokines.
Materials and methods: Our hypothesis about regulatory network of Tregs and NKT cells was studied by flow cytometry, analyzing the characteristics of human Tregs and NKT cells, by phenotypic analysis of peripheral blood lymphatic cells of inhabitants leaving near gasoline industry, compared to people from rural areas, as well as by cytotoxicity assay against tumor cell line (human NK-sensitive line K562) to determine the functions ofcytotoxic T lymphocytes and natural killer (NK) cells, which have a crucial role in innate immunity.
Results: Our data have shown that inhabitants living near industrial area have significantly increased percentage of NKT and Tregs in comparation with those from rural fields, which may interplay with the function of immune system and percentage of allergic diseases. Interestingly, all exposed individuals have significant diminished values of NK-mediated cytotoxicity against human NK-sensitive target in comparation with people from rural area.
Conclusions: It has been proposed that NKT cells perform an important subpopulation of cells which can play both roles: as effectors and as regulatory cells in a wide range of disease settings. We can talk about the new kind of cells: NKT-reg cells. NKT-reg cells monitoring may lead to important early diagnosis and/or prognosis of environmental diseases.
Acknowledgement: This work was supported by grants from the Croatian Ministry of Science (No. 062-0621341-0308, 062-0620096-0094 and 062-0621341-1337).

P0653
TCR requirements for gamma delta T cell development

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Purpose/Objective: Gamma delta ($\gamma\delta$) T cells play important roles in immune responses against pathogens and tumours which seem to

strongly correlate with the secretion of cytokines such as IFNγ and IL-17A. Recent evidence suggests that $\gamma\delta$ cells are predominantly pre-committed to certain effector fates during thymic development; T cell receptor (TCR)-agonists favouring the development of IFNγ-producing $\gamma\delta$ cells (that are variously identified by CD27, CD122 and NK1.1), whereas the absence of ligand interactions was suggested to generate IL-17A-producing $\gamma\delta$ cells (identified as CD27$^{(-)}$, but expressing CCR6). The aim of this study was to determine the role of the TCR and TCR signalling in the development of $\gamma\delta$ T cell subsets and in the adoption of effector fates in the thymus.
Materials and methods: Integrating all the markers described above including CD24 and CD25, we used flow cytometry to establish a gating strategy to identify IFNγ-secretors and IL-17A-secretors in the lymph nodes and thymus of C57BL/6 (BL/6) mice. Subsequently, we sorted various $\gamma\delta$ subsets and put them into different culture systems including fetal thymic organ cultures (FTOC) as well as on to OP9-DL1 stromal cell line to analyse $\gamma\delta$ cell development and the adoption of different effector functions under different conditions. These conditions included inducing TCR signalling by cross-linking with an activating antibody or in a ligand-independent manner by generating a TCR that was incapable of binding a ligand.
Results: Our results demonstrate that at least four distinct subsets of $\gamma\delta$ T cells can be identified in both the periphery and the thymus of BL/6 mice. These subsets display distinct cytokine-secreting and proliferative potential as well as variable TCR usage. Our data *in vitro* demonstrate a $\gamma\delta$ developmental sequence where CD27$^{(-)}$ IL-17A-secreting $\gamma\delta$ T cells are generated from CD27$^{(+)}$ precursors. In addition, we find that TCR signalling induced by cross-linking with an activating antibody or in a ligand-independent manner does not favour the development of IL-17A-secreting $\gamma\delta$ T cells. Furthermore, we observed differences in downstream signalling events between the $\gamma\delta$ subsets analysed.
Conclusions: We conclude that effector fate of $\gamma\delta$ subsets is not likely to correlate with ligand-dependent versus ligand-independent signalling but instead appears to correlate with quantitative and/or qualitative differences in TCR signalling.

P0654
The impact of Skint genes on peripheral gammadelta T cell function

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Purpose/Objective: Skint1 expressed by thymic epithelial cells is essential for the selective development of murine dendritic epidermal T cells (DETCs), which express an identical Vγ5Vδ1 T cell receptor (TCR). Mice lacking V$\gamma5^+$ V$\delta1^+$ DETCs display dysregulated cutaneous inflammation and an increased susceptibility to carcinogenesis. The function of Skint1 on keratinocytes is unknown and it has yet to be determined whether Skint1 is a ligand for the Vγ5Vδ1 TCR. A recent study demonstrated a sustained interaction between Vγ5Vδ1 TCRs localised in the termini of immobilised DETC dendrites and an unknown ligand expressed on keratinocytes, which resulted in constitutive signalling downstream of the TCR [and the formation of phosphotyrosine-rich aggregates located on projections (PALPs)]. We are investigating the contribution of Skint1 to this steady state interaction, the role of Skint2 in the selection of DETCs and other $\gamma\delta$ T cell repertoires e.g. uterine intraepithelial T cells and the regulation of-Skint1 and Skint2 in response to skin stresses.
Materials and methods: To investigate the role of Skint1, we used confocal microscopy to compare the number of PALPs in strains of FVB mice differentially expressing Skint1 and/or the Vγ5Vδ1 TCR, before and after skin stress. Skint mRNA levels were analysed by RT-PCR. To investigate the role of Skint2, we disrupted Skint2 gene functions by replacing it with a fluorescent reporter.
Results: DETCs from mice in which Skint1 (and therefore also V$\gamma5^+$ V$\delta1^+$ DETCs) was absent and DETCs from Vγ5V$\delta1^{-/-}$ mice, had fewer PALPs than DETCs from wild-type mice and mice transgenic for Skint1. Following application of low concentrations of TPA to WT mice, Skint1 mRNA levels in the epidermis decreased and the DETCs resembled those from mice lacking Skint1.
Conclusions: Hence Skint1 has a profound effect on the peripheral status of DETCs. The results of further experiments looking at the effect of low concentration TPA on Skint1 transgenic mice (in which the Skint1 mRNA levels remain constant) and the role of Skint1 in keratinocytes specifically will be reported, as will the progress resulting from experiments using the Skint2 knockout and reporter mice.

P0655
The impact of Skint genes on peripheral gammadelta T cell function

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Purpose/Objective: Skint1 expressed by thymic epithelial cells is essential for the selective development of murine dendritic epidermal T cells (DETCs), which express an identical Vγ5Vδ1 T cell receptor (TCR). Mice lacking V$\gamma5^+$ V$\delta1^+$ DETCs display dysregulated cutaneous inflammation and an increased susceptibility to carcinogenesis. The function of Skint1 on keratinocytes is unknown and it has yet to be determined whether Skint1 is a ligand for the Vγ5Vδ1 TCR. A recent study demonstrated a sustained interaction between Vγ5Vδ1 TCRs localised in the termini of immobilised DETC dendrites and an unknown ligand expressed on keratinocytes, which resulted in constitutive signalling downstream of the TCR [and the formation of phosphotyrosine-rich aggregates located on projections (PALPs)]. We are investigating the contribution of Skint1 to this steady state interaction, the role of Skint2 in the selection of DETCs and other $\gamma\delta$ T cell repertoires e.g. uterine intraepithelial T cells and the regulation of-Skint1 and Skint2 in response to skin stresses.
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Results: DETCs from mice in which Skint1 (and therefore also V$\gamma5^+$ V$\delta1^+$ DETCs) was absent and DETCs from Vγ5V$\delta1^{-/-}$ mice, had fewer PALPs than DETCs from wild-type mice and mice transgenic for Skint1. Following application of low concentrations of TPA to WT mice, Skint1 mRNA levels in the epidermis decreased and the DETCs resembled those from mice lacking Skint1.
Conclusions: Hence Skint1 has a profound effect on the peripheral status of DETCs. The results of further experiments looking at the effect of low concentration TPA on Skint1 transgenic mice (in which the Skint1 mRNA levels remain constant) and the role of Skint1 in keratinocytes specifically will be reported, as will the progress resulting from experiments using the Skint2 knockout and reporter mice.

P656
The role of $\gamma\delta$ T cells in *Porphyromonas gingivalis*-induced periodontal disease

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Purpose/Objective: $\gamma\delta$ T cells are a rare population of T lymphocytes found in most tissues that play an important but poorly defined role in protective immunity to infection. $\gamma\delta$ T cells appear to have both

proinflammatory and regulatory functions, acting as a bridge between the innate and adaptive immune response as well as down modulating the inflammatory response once an infection is cleared. Chronic periodontitis is an inflammatory disease of the supportive tissues of the teeth leading to resorption of alveolar bone and eventual tooth loss. The disease is a major public health problem in all societies and is estimated to effect 5−20% of any population. Recent studies are now strongly indicating that chronic periodontitis effects systemic health and is a major risk factor for diseases such as coronary heart disease, pancreatic cancer, diabetes and rheumatoid arthritis. Whilst several bacterial species are strongly associated with disease, *Porphyromonas gingivalis* has been implicated as a major etiological agent.

Materials and methods: We investigated the role $\gamma\delta$ T cells have in chronic periodontitis using the mouse bone loss model, ELISA and ELISPOT assays. We also isolated $\gamma\delta$ T cells (autoMACS assay) and determeined their activation state and secretion of IL-17 and IFNγ by flow cytometry and ELISPOT.

Results: In a mouse model of periodontitis the absence of $\gamma\delta$ T cells (TCR$\delta^{-/-}$ mice) resulted in significantly ($P < 0.05$) less *P. gingivalis* induce bone loss compared to wild type controls. In the *P. gingivalis*-inoculated TCR$\delta^{-/-}$ mice there was a *P. gingivalis*-specific antibody and $\alpha\beta$ T cell response albeit weaker than *P. gingivalis*-inoculated control. In the *P. gingivalis*-inoculated TCR$\delta^{-/-}$ mice there was a weak and equal IL-4 and IFNγ $\alpha\beta$ T cell response, whereas in the *P. gingivalis*-inoculated control mice a IFNγ $\alpha\beta$ T cell response was dominant. Furthermore *P. gingivalis* whole cells and lipids were found to up regulate CD69 and CD25 expression on $\gamma\delta$ T cells and induce IL-17 and IFNγ secretion.

Conclusions: Our study strongly indicates that $\gamma\delta$ T cells play a major role in periodontitis and that they are directly activated by *P. gingivalis* and its lipids, resulting in the secretion of pro-inflammatory cytokines which may contribute to disease pathology

P0657

Thymus and major histocompatibility complex class II-dependent CD8alpha/beta$^+$ T cell receptor gamma/delta intestinal intraepithelial lymphocytes in mice

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Purpose/Objective: In the mouse, intestinal epithelial layers constitute a rich source of T cell receptor (TCR) γ/δ cells and the vast majority of TCRγ/δ intestinal intraepithelial lymphocytes (iIEL) surface express CD8α, but lack CD8β. In the present study, we examined whether functional TCRγ/δ cells expressing both CD8α and CD8β exist in the small intestine and whether the development of this cell population is controled by major histocompatibility complex (MHC) class I or MHC class II.

Materials and methods: We examined whether TCRγ/δ cells expressing both CD8α and CD8β exist in the small intestine by flow cytometry and whether the development of this cell population is controlled by MHC class I or MHC class II using various mouse strains including MHC class I or class II-deficient mice. Moreover, we also examined whether this cell population expresses functional activities such as interferon (IFN)-γ-secreting activity and cytolytic activity.

Results: We identified intestinal TCRγ/δ cells expressing both CD8α and CD8β in some mice. These CD8α/β^+ TCRγ/δ iIEL emerge in C57BL/6 (H-2^b) mice independently from age and bacterial colonization, although their abundance varied among individual animals. CD8α/β^+ TCRγ/δ cells were restricted to the intestine and virtually absent in other organs. CD8α/β^+ TCRγ/δ iIEL were rare in adult and neonatally thymectomized C57BL/6 mice. Substantial numbers of CD8α/β^+ TCRγ/δ iIEL were found in mice deficient for β2-microglobulin (H-2^b), whereas they were rare in mice deficient for MHC class II (H-2^b) and also in C57BL/10 (H-2^b), SVJ129 (H-2^b), BALB/c (H-2^d), C3H/HeJ (H-2^d), DBA/2 (H-2^d), CBA (H-2^k), SJL (H-2^s) and FVB (H-2^q) mice. Profound IFN-γ-secretion and target cell lysis were found in iIEL from C57BL/6 mice comprising unconventional CD8α/β^+ TCRγ/δ iIEL as compared to C57BL/6 mice lacking these cells. Purified CD8α/β^+ TCRγ/δ iIEL from C57BL/6 mice expressed elevated cytolytic activities and IFN-γ after TCRγ/δ engagement.

Conclusions: Our findings suggest that emergence of CD8α/β^+ TCRγ/δ iIEL with functional activities is controlled by thymic epithelial cells and by MHC class II-related molecules distinct from H-2^b.

P0658

Transcriptional and epigenetic signatures of pro-inflammatory gamma-delta T cell subsets

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Purpose/Objective: Interferon-gamma (IFN-γ) and interleukin-17 (IL-17) are critical pro-inflammatory cytokines which play protective roles during host defense against infections, but also, pathogenic roles such as during autoimmunity.

Gamma-Delta ($\gamma\delta$) T cells are major early sources of IFN-γ and IL-17. We identified two murine subsets of $\gamma\delta$ T cells that segregate with CD27 expression and display mutually exclusive capacities to make IFN-γ or IL-17. These phenotypically and functionally distinct populations differentiate already within the thymus and colonize peripheral lymphoid organs.

Our objective is to get insights into the molecular basis of the developmental pre-programming that controls the emergence of these discrete cytokine-producing $\gamma\delta$ T cell subsets.

Materials and methods: The acquisition of selective effector functions relies on specific molecular programs that tightly control the chromatin structure (at an epigenetic level) and transcriptional activity of a combination of genes. Thus, here we have undertaken:

- an unbiased approach to analyze active histone H3 marks (methylation at K4)

- a candidate gene approach to evaluate the expression of key transcription factors

This led to mapping the key transcription factor and cytokine expressions, as well as active histone (H3K4me2) marks in FACS-sorted CD27$^+$ and CD27- $\gamma\delta$ T cells from naïve C57Bl/6 mice.

Results: - Active histone H3K4 methylation marks in type 17 genes are differentially distributed between CD27$^+$ and CD27- $\gamma\delta$ T cells and ensure a striking restriction of *Il17* expression to CD27- $\gamma\delta$ T cells. By contrast, type 1 gene loci are epigenetically poised in both subsets.

- Key type 1 and type 17 transcription factors (including Tbox21, Eomes, Runx3, or RORc, RORα) are expressed at similar levels in both CD27$^+$ and CD27- $\gamma\delta$ T cell subsets derived from the thymus. By contrast, CD27$^+$ and CD27- $\gamma\delta$ T cells derived from secondary lymphoid organs show significant differential expression of the type 17 genes.

- Upon 48h *in vitro* re-stimulation, in the presence of cytokines, CD27- $\gamma\delta$ T cells become able to secrete both IL-17 and IFN-γ, while, CD27$^+$ $\gamma\delta$ T cells only produce IFN-γ.

Conclusions: Our results provide novel insight into the mechanisms that control the programming and plasticity of functional $\gamma\delta$ T cell subsets.

Poster Session: Regularity T Cell Subsets and Plasticity

P0660

B7-CD28 and CD40-CD40L blockade leads to hypo-responsiveness of effector T cells but allows for activation of Foxp3-expressing regulatory T cells

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Purpose/Objective: Costimulatory signals are required for efficient priming and activation of naïve T cells, while it is less clear how they contribute to induction of regulatory T cell (Treg) activity. We previously reported that the simultaneous and prolonged blockade of B7-CD28 interaction and of the CD40L-CD40 interaction *in vivo* efficiently suppresses allogeneic T cell activation after transfer of spleen cells to an allogeneic host. This protection was characterized by an initial rise in Foxp3$^+$ cells (without *de novo* induction), followed by disappearance of host reactive cells.
Materials and methods: To further investigate the effects of costimulatory blockade on Treg cells, we used an *in vitro* model of CD4 cell activation with allogeneic splenocytes. In order to faithfully track Foxp3-expressing cells, we used cells from Foxp3-GFP transgenic mice. The CD28-CD80/CD86 and the CD40-CD40L pathways were blocked using CTLA-4Ig and MR1 (anti-CD40L mAb), respectively.
Results: We show that blocking B7 molecules (using CTLA4-Ig) together with CD40L (using anti-CD40L mAb) prevents effector T cell activation and proliferation. Moreover, we observed a relative increase in Foxp3 expression in cultures with costimulation deficient conditions, which does not result from *de novo* induction of Foxp3 but rather from proliferation of Treg cells and their ability to survive in an environment with limited IL-2 availability. Furthermore, we show that cells cultured in the presence of costimulation blockade are hyporesponsive upon re-stimulation unless Foxp3 expressing cells are removed. Under costimulation deficient conditions activated Foxp3 expressing Treg cells are further able to prevent T cell proliferation *in vitro* in the absence of costimulation blockade more efficiently than freshly isolated Treg cells.
Conclusions: Together these results suggest that Treg cells are less dependent on CD40L and B7 signaling concerning activation, survival and proliferation. As a consequence, they become functionally dominant over effector T cells in a situation of costimulation blockade.

P0662

Clinical significance of NKT and Treg cells in autoimmune thyroid disease during pregnancy

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Purpose/Objective: NKT cells represent a unique sublineage of innate lymphocytes, that share the properties of natural killer cells and conventional T cells. Autoimmune thyroid dysfunction (ATD), which comprises two main clinical entities: Graves' disease and Hashimoto thyroiditis, often affect women of reproductive age. In a healthy pregnancy predominant is Th2 over Th1 immunity, which explains the improvement of autoimmune disease during pregnancy, while after birth due to changes in Th1/Th2 ratios often leads to deterioration of ATD. NKT and Tregs seem to play an important part in mediating maternal tolerance to fetus. Although many researches have been done in the field ofthyroid autoimmunity, very few studies investigated the role of innate immunity in AITD during human pregnancy and in the postpartal period.
Materials and methods: We investigated the presence of ATD in pregnant and postpartum period in women with hormonal status determination, the titer of thyroid antibodies and auto antibodies and compared them with healthy pregnant women and subjects' postpartum and not pregnant women. After intracellular and surface staining using flow cytometry, we analyzed the phenotype and cytolytic potential of isolated peripheral blood mononuclear cells of pregnant women and postpartum women, and not pregnant women.
Results: Total in 25% of pregnant women and 41.5% of postpartum women have demonstrated the presence of thyroid autoimmunity. The cells of innate immunity: NKT and Tregs are elevated in healthy pregnancies and in pregnancies with hypo-and hyperthyroidism, as well as postpartum in all experimental groups. NKT cells are reduced in pregnant women with positive antibodies indicating a decrease in NKT cell protective effect of pregnancy under the action of antibodies. Total perforin was decreased in healthy women, but higher in patients with positive antibodies, hypo-and hyperthyroidism and postpartum indicating the increased cytolytic potential of these cells in autoimmune process.
Conclusions: Pregnancy and the postpartum period influence the function of the thyroid gland. In the presence of thyroid autoimmunity changes are more pronounced, especially postpartum. Apart from pregnancy and postpartum period influence the course of ATD and thyroid autoimmunity affects thyroid function in pregnancy and the postpartum period.

P0663

Constitutively active STAT3 in T-cells induces a distinct tolerogenic program

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Purpose/Objective: The signal transducer and activator of transcription 3 (STAT3) is a key integrator of signals provided by a multitude of cytokines and factors. Yet, the role of STAT3 in T-cells is not well established. Current research indicates Th17-inducing but also tolerogenic roles. We assessed the effects of STAT3 activation in human T-cells.
Materials and methods: A constitutively active mutant of STAT3 (STAT3C) was generated and cloned into an IRES-GFP harboring retroviral vector. Upon transduction into peripheral blood CD4$^+$ T-cells we analyzed phenotype, cytokine secretion, proliferation and suppressive potential in response to qualitatively different stimuli.
Results: STAT3C$^+$ T-cells showed clear-cut transgene expression and phenotypically displayed an effector T-cell phenotype regarding the assessed characteristic regulatory T-cell markers CD25, CD39, CD127 and CTLA-4. Following anti-CD3 stimulation in concert with costimulatory signals via either CD80-CD28 or CD58-CD2, STAT3C$^+$ T-cells were hyporesponsive and displayed a distinct cytokine profile. This included a significant reduction of IL-2, IL-13, IFN-γ and TNF-α secretion and a fourfold increase in IL-10 production. In co-cultures with CD4$^+$ responder T-cells, STAT3C$^+$ T-cells showed no suppressive capacity in response to CD80 co-stimulation but significant suppressive capacity following CD58 co-stimulation. Suppression was contact-dependent but independent of IL-10 and TGF-β. Preliminary microarray data suggest that STAT3C$^+$ T-cells up-regulate a distinct set of immunoregulatory genes.

Conclusions: Our data provide a clear-cut relation between activation of STAT3 and the acquisition of an anti-inflammatory cytokine profile and suppressive function. Consequently, the main function of STAT3 in T-cells seems to be the regulation of tolerogenic processes.

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P0664
Deficiency of glycoprotein A repetitions predominant in T cells predisposes to severe colitis

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Purpose/Objective: Glycoprotein A repetitions predominant (GARP) is a transmembrane protein with an extracellular region composed primarily of 20 leucine-rich repeats. It belongs to orphan Toll-like receptors and is expressed in different cells and organs during embryogenesis. Recently, it has been shown that GARP expression is highly upregulated in human activated natural regulatory CD4 T cells (nTreg). The function of this protein is still unknown, although some studies suggest that GARP might be necessary for the activation of TGF-§ in Tregs upon TCR stimulation.
Materials and methods: To evaluate the function of GARP in Tregs we generated mice lacking GARP in T cells using a Cre-Lox conditional gene targeting approach. Flow cytometry was used to estimate the phenotypical changes in mice deficient for GARP in T cells. The dextran sodium sulfate (DSS) model of colitis was used to assess the role of GARP in a suppressive function of Tregs in inflammatory conditions.
Results: The conditional deletion of GARP resulted in decreased frequencies of CD4 T cells in the blood, spleen and lymph nodes, whereas frequencies of $CD69^+$ /$CD4^+$ cells were increased. Analysis of the small intestine revealed increased numbers of CD4 T cells and a shift in the ratio of TCRab/TCRgd T cells towards TCRab in the lamina propria and the intraepithelial layer. Frequencies of $Foxp3^+$ CD4 T cells were slightly increased in the spleen, mesenteric and inguinal lymph nodes, whereas in the gut their frequencies were significantly decreased. Mice with conditional deletion of GARP developed more severe colitis assessed by clinical and histological scores. Treatment of GARP-deficient mice with nTregs from wild type mice before induction of colitis resulted in amelioration of disease activity, whereas GARP-deficient nTregs failed to improve.
Conclusions: These data demonstrate that selective knock out of GARP in T cells alters CD4 T cell subset distribution and results in higher susceptibility to colitis. This might be related to the decreased ability of Tregs to migrate into the gut.

P0665
Diacylglycerol kinase alpha is essential for regulatory T cell induction

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Purpose/Objective: T cell receptor triggering in T cells leads to generation of diacylglycerol (DAG), a lipid second messenger that mediates activation of the RasGRP1/Ras/Erk pathway. Diacylglycerol kinase alpha (DGKα) catalyzes DAG conversion to phosphatidic acid (PA), and negatively regulates this pathway during $CD4^+$ T cell activation. Activated $CD4^+$ T cells then differentiate further into distinct populations. The fate of naïve T helper (Th) cells as effector or regulatory cells is linked to activation signal intensity and regulated by mTOR. We analyzed the role of DGKα in Treg cells and the impact of DGKα deficiency in T effector versus Treg cell determination.
Materials and methods: To study the implication of DGKα in induced Treg cell (iTreg) differentiation, $CD4^+$ $CD25^-$ naïve T cells were stimulated with anti-$CD3^+$ anti-CD28 antibodies and treated with TGFβ and IL2, followed by confirmation of Treg induction by flow cytometry analysis of FOXP3 expression. DGKα expression was determined by Western blot analysis with specific antibodies. To understand the impact of DGKα deficiency in Treg cells, we compared the proportion of iTreg as well as of natural Treg cells in DGK$\alpha^{-/-}$ and wild type mice.
Results: We found that at difference from T effector cells, iTreg express high DGKα levels. Sustained DGKα expression is important for maintenance of the regulatory phenotype, as FOXP3 induction is greatly impaired in DGK$\alpha^{-/-}$ compared to WT mice.
Conclusions: Our results suggest that sustained DGKα expression is important for attenuation of the TCR signals needed for differentiation of Th cell populations. Enhanced activation of the Ras/ERK/mTOR pathways in DGK$\alpha^{-/-}$ mice prevents iTreg cell differentiation. The contribution of DGKα to T effector versus Treg differentiation will be discussed.

P0666
Differential quantitative mass spectrometry of the treg proteome

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Purpose/Objective: In the past few years, regulatory T cells (Treg) have emerged as important players of the immune system. Impaired function of Treg cells leads to several autoimmune diseases. In addition, they are involved in regulating immune homeostasis and the immune response to infections, tumors and tissue transplants. Therefore, a better understanding of Treg cell function and development is crucial. Previously, we and others performed gene expression profiles of Treg cells under a variety of conditions and locations. But, so far, only limited data exist on the proteome level. Thus, we addressed the proteome of Treg cells in an unbiased differential quantitative mass spectrometry approach.
Materials and methods: Proteins from sorted murine Treg cells and conventional $CD4^+$ T cells were differentially labeled with stable-isotopes, joint together and relative protein abundance was measured by differential quantitative mass spectrometry. To increase the power, four independent replicates were performed and the results were statistically analyzed.
Results: Mass spectroscopy identified and quantified more than 4000 proteins of which 165 were significantly differentially expressed in Treg cells as compared to conventional $CD4^+$ T cells. In depth analysis of the obtained proteome data and corresponding genomic data sets revealed an overall strong correlation between protein and RNA expression. For example, some of the Treg specific genes like Foxp3, IL2rβ, GITR or neuroplin-1 were highly over-expressed on both protein and RNA level. In addition, we identified several novel proteins which were under- or over-represented on the protein level as compared to the RNA level.
Conclusions: In gereral protein expression follows RNA but some interesting exceptions occur. We are now in the course of investigating the role of targets in Treg function and development.

P0667

DSS-induced colitis is attenuated in GPx1 KO mouse by regulation of treg function

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Purpose/Objective: Reactive oxygen species (ROS) have been considered harmful to tissues and as mediators of inflammation at the injury sites for a long time. However, in recent, many findings about other functions of ROS are reported. The effects of ROS such as suppression of autoimmune disease and allergen-induced inflammation have been studied by using knock-out systems for ROS-related molecules. Moreover, regulatory T cell (Treg) effects to various diseases including autoimmune and inflammatory diseases depending on own activity. Therefore, we tried to identify the relation of Treg function with ROS in chemical induced inflammation through glutathione peroxidase 1 knock-out (GPx1 KO) system.
Materials and methods: C57BL/6 WT mice and GPx1 KO mice were used for entire experiments. Colitis was induced by 3% dextran sulfate sodium salt (DSS) and the colons were excised. We detected losses of weight, colon lengths by macroscopic anatomy and histological scores by hematoxylin & eosin staining. Treg functional tests were performed by CFSE labeling and cell proliferation assay. Some cytokines in culture supernatants of mice splenocytes were detected by cytometric bead array.
Results: First of all, losses of weight were founded in not GPx1 KO mice but B6 WT mice by DSS. And then, macroscopic inspection clearly showed colon shortening in DSS-WT mice and NAC injected DSS-GPx1 KO mice. In histological data, colon tissues of DSS-Gpx1 KO mice had normal structure whereas those of DSS-WT mice showed abnormal lesions such as ulcer, inflammation and early adenocarcinoma. Furthermore, suppressive activity of regulatory T cell was diminished in DSS-WT mice and it had correlation with cytokine distribution.
Conclusions: These data indicate that ROS accumulation in GPx1 KO mice activates regulatory T cells and DSS-induced colitis is controlled by Treg depending on own activity.

P0668

Feasibility of using regulatory T cells as an 'off the shelf' cellular reagent

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Purpose/Objective: Treg offer a potential therapeutic modality for the prevention and/or treatment of autoimmune disorders and protection of adverse effect following allogeneic transplantation. Several clinical trials using freshly isolated or *ex vivo* expanded Treg have shown promising therapeutic benefit creating an opportunity for cryopreserved Treg to be used as an 'off the shelf' cellular reagent either in the clinic or for research purposes. This study aimed to investigate whether Treg retain their suppressive properties after cryopreservation.
Materials and methods: $CD4^+$ $CD25^{hi}Foxp3^+$ Treg were isolated from LRS cones followed by *ex vivo* expansion in the presence of anti-CD3/CD28 beads, rapamycin and IL-2. In comparison to non-cryopreserved Treg, the suppressive properties of cryopreserved Treg were assessed for their ability to modulate the alloreactive immune responses of $CD8^+$ T cells, including activation, proliferation, cytokine production and prevention of cutaneous graft-versus-host reactions, using flow cytometry, ^3H incorporation, CBA and a skin explant assay.
Results: Large number of Treg with high suppressive potency was obtained. Foxp3 expression and suppressive function of Treg remained stable for over 5 weeks in *ex vivo* expansion culture. The inhibition rate of alloreactive $CD8^+$ T cell proliferation was 77% and 79% for Treg with or without cryopreservation respectively. Early activation marker CD69 was suppressed by 71% and 76% and late activation marker CD25 expression was suppressed by 83% and 84% for freshly expanded and cryopreserved Treg respectively. The potency of Treg suppression in IFNγ production by alloreactive $CD8^+$ cells was comparable regardless of cryopreservation. Furthermore, cryopreserved Treg retained their protective effect on alloreactive $CD8^+$ T cell mediated target tissue destruction in an *in vitro* human GVHD skin explant model.
Conclusions: No significant difference was observed between the suppressive properties of Treg with or without cryopreservation, when they were used to modulate allogeneic moDC stimulated $CD8^+$ T cells immune responses, suggesting that Treg suppressive functions are not affected by cryopreservation and could potentially be developed as an 'off the shelf' cellular reagent.

P0669

$Foxp3^+$ follicular regulatory T cells control the germinal centre response

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Purpose/Objective: T Follicular helper (Tfh) cells provide growth and survival signals to germinal centre B cells as they undergo the process of somatic hypermutation and selection that results in affinity maturation. Tight control of the Tfh population is vital to maintain self-tolerance and ensure that self-reactivity does not arise from the germinal centre. Here, we describe a population of $Foxp3^+$ $Bcl6^+$ $CD4^+$ T cells that also share cell surface markers with Tfh cells and constitute 10–25% of the $CXCR5^{high}PD\text{-}1^{high}CD4^+$ T cell population found in splenic germinal centres after immunisation.
Materials and methods: We immunized wild-type, $Foxp3^{GFP}$, $Foxp3^{DTR}$, *Sh2d1a*$^{-/-}$, *Cd28*$^{-/-}$ and *Bcl6*$^{-/-}$ mice, or mixed bone marrow chimeras of these genotypes, to investigate the phenotype of follicular regulatory T (Tfr) cells, to determine whether they derive from Treg or Tfh precursors, what cues are required for their differentiation and ascertain what their role is in the germinal centre response.
Results: Follicular regulatory T (Tfr) cells share phenotypic characteristics with both Tfh and conventional $Foxp3^+$ regulatory T cells (Tregs) yet are distinct from either. Similar to Tfh cells, Tfr cell development depends on the expression of Bcl-6, the transcriptional regulator of the Tfh subset, furthermore their maintenance requires SAP-mediated cognate interactions with B cells. This shared differentiation pathway suggests that Tfr cells may arise from Tfh cells that have switched on Foxp3; however Tfr cells originate from $Foxp3^+$ precursors and not naïve T cells or Tfh cells.This demonstrates that Tregs can co-opt the Tfh differentiation pathway to migrate into the germinal center, where they can participate in the response. Tfr cells are suppressive *in vitro* and limit Tfh and germinal centre numbers *in vivo*. In the absence of Tfr cells, there is an outgrowth of non-antigen-specific B cells in germinal centres leading to a reduced number of antigen-specific germinal centre B cells.

Conclusions: Our results indicate that Foxp3$^+$ Tregs can utilise the Tfh differentiation pathway to produce a population of specialised suppressor cells that control the size and composition of the germinal centre response.

P0670

Foxp3$^+$ regulatory T cells in the inflamed CNS are insensitive to IL-6-driven IL-17 production

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Purpose/Objective: Foxp3$^+$ T regulatory cells (Treg) can be induced to produce IL-17 by *in vitro* exposure to pro-inflammatory cytokines drawing into question their functional stability at sites of inflammation We sought to assess the capacity of Treg from an inflammatory site produce IL-17 in response to IL-6.

Materials and methods: Purified Foxp3$^+$ Treg from the inflamed central nervous system (CNS) of mice with experimental autoimmune encephalomyelitis and splenic Treg were stimulated *in vitro* in the presence of IL-6 and their capacity for IL-17 production determined.

Results: Unlike their splenic counterparts, CNS-Treg resisted conversion to IL-17 production when exposed to IL-6. The highly activated phenotype of CNS-Treg includes elevated expression of the Th1-associated molecules CXCR3 and T-bet, but reduced expression of the IL-6 receptor α chain (CD126) and the signalling chain gp130. We found a lack of IL-6 receptor on all CNS CD4$^+$ T cells, which was reflected by an absence of both classical and *trans* IL-6-signalling in CNS CD4$^+$ cells, compared to their splenic counterparts.

Conclusions: Lack of responsiveness to IL-6 stabilizes the regulatory phenotype of activated Treg at sites of autoimmune inflammation. Increased knowledge of cytokine receptor expression profiles will help predict the responsiveness of different T cell subsets to inflammatory environments.

P0671

Functional change of regulatory T cells during an experimental infection with sparganum (plerocercoid of Spirometra mansoni)

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Purpose/Objective: Regulatory T cells (Tregs) are important in the regulation of immune response, but how Treg function is actually regulated *in vivo* is still not well known. In order to understand how Treg function is regulated during a parasite infection, we investigated the functional activity of CD4$^+$ CD25$^+$ Tregs as well as the frequency and number of CD4$^+$ CD25$^+$ FoxP3$^+$ Tregs in the spleens of mice experimentally infected with sparganum (plerocercoid of *Spirometra mansoni*) for 3 weeks..

Materials and methods: Male BALB/c mice were infected with 2 spargana and were sacrificed at day 3, week 1, 2, and 3 for the functional study of spleen CD4$^+$ CD25$^+$ Tregs, CD4$^+$ CD25$^-$ effector T cells (Teffs), and CD4$^-$ antigen-presenting cells (APCs), by using CFSE dilution assay. When necessary, crude extracts (CE) or excretory-secretory products (ESP) of sparganum were added for the study of host-parasite interaction. Cytokine-producing capability of the splenocytes was investigated by restimulation assay *in vitro* and measurement of cytokine levels in the supernatants by cytometric bead array, in order to study the pattern of immune response of the mice to the parasite. Specific antibody to spargana was measured in the sera by ELISA, as an objective parameter of adaptive immune response to the parasite.

Results: Treg function fluctuated during the parasite infection, being upregulated at day 3, downregulated until week 2, and thereafter upregulated again in week 3. The capabilities of the splenocytes to produce IL-2, IFN-g and IL-17a were decreased, while those of IL-4 and IL-10 were increased along the time course of the parasite infection. Meanwhile, IL-6 producing capability was increased to reach a peak at week 2, and thereafter was decreased to the baseline level. As a regulatory mechanism, we found Tregs proliferated well *in vitro*, in the presence of IL-2, and in particular to weak stimulating signals better than CD4$^+$ CD25$^-$ Teffs. We also found that the CE decreased, whereas the ESP of sparganum increased the suppressive function of Tregs, suggesting that sparganum products were involved in the triggering and regulation of immune response in the acute and chronic phases, respectively.

Conclusions: Taken together, Tregs are central in the immune homeostasis *in vivo* maintained by host-parasite interaction during the parasite infection.

P0672

Functional roles for defective PI3K signaling and differing co-stimulatory activity of APCs in T-Cell plasticity

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Purpose/Objective: The activation of naïve CD4$^+$ T-cells by naïve peptide loaded B cells (TofB) leads to the production of T cells with a CD62-L high phenotype and regulatory properties (Treg) *in vivo* and *in vitro*. This is in contrast with the functional outcome following activation of T-cells with dendritic cells serving as APC (TofDC). We sought to investigate the underlying molecular mechanisms governing this differential regulation of CD62-L and the induction of a regulatory phenotype.

Materials and methods: 8–12 weeks old OT-2 and DO11.10 were sources of naïve CD4 T-cells specific for a peptide of chicken ovalbumin. Balb/c and C57/Bl6 mice were used as sources of DC and naïve B-cells. Splenic T and B-cells were negatively enriched using the MACS system. DC were obtained from bone marrow precursors differentiated with GM-CSF and IL4 or GM-CSF alone and treated with LPS when further functional maturation should be induced.

Results: Inhibition of PI3K/Akt and mTOR signaling led to increased CD62-L expression in the TofDC. Signal profiling of the differentially activated T-cells revealed a specific defect in the PI3K signaling pathway. In the TofB, we identified an attenuated phosphorylation of the hydrophobic motif of Akt occurring at the time of divergence in CD62-L kinetics between TofB and TofDC. T-cells triggered with immature DCs which are known to tolerize T-cells also induced an attenuated Akt profile in addition to a high CD62-L phenotype, which were both indistinguishable from the effects seen in TofB. Under certain conditions, it was also possible to induce regulatory behavior in T-cells triggered with matured DCs via fine tuning of the P13K but not the mTOR pathway.

Conclusions: Thus, the regulation of CD62-L involves bothPI3K/Akt and mTOR signaling; however the PI3K/Akt axis seems to be singularly responsible for the induction of a regulatory phenotype.The applica-

tion of this knowledge might be useful for tuning immune responses i.e. for inducing Treg production in cases of exaggerated immune responses or vice-versa.

P0673

HLA-C KIR-ligand allotype differentially induces the expansion of alloreactive CD8$^+$ KIR2D$^+$ T lymphocytes *in vivo* and *in vitro*

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Purpose/Objective: Results from our group have shown better early liver allograft acceptance associated with *in vivo* expansion of CD8$^+$ KIR2D$^+$ T cells. Apparently, HLA-C differentially influences the expansion of CD8$^+$ KIR2D$^+$ T cells with C1C1 and C1C2 but not C2C2 HLA-C KIR-ligand allotypes showing clear expansions 1 year after transplantation. The purposes of this study was to confirm in an *in vitro* model the differential expansion of CD8$^+$ KIR2D$^+$ responder T cells according to their HLA-C KIR-ligand allotype, and characterize the functional profile of expanded cells.
Materials and methods: Three different set of allogenic cultures were carried out in parallel using PBLs from healthy individuals. C1/C2-heterozygous stimulator cells were used to stimulate C1C1, C1C2 and C2C2 responder cells during 20-days. The cytotoxic capability of CD3$^+$ CD8$^+$ KIR2D$^+$ expanded cells was tested using the CD107a degranulation assay and a LSR-II flow cytometry (B. Dickinson).
Results: CD8$^+$ KIR2D- T-cells showed similar expansion for all HLA-C KIR-ligand allotypes. In contrast, C2C2-allotype showed significantly lower expansion of CD8$^+$ KIR2D$^+$ T cell than C1-allotypes.CD8$^+$ KIR2DL1/S1$^+$ T cells expanded by 39, 20 and 5.8 for C1C1, C1C2 and C2C2, respectively ($P < 0.05$). CD8$^+$ KIR2DL2/L3/S2$^+$ T cells expanded by 36, 21 and 12 for C1C1, C1C2 and C2C2, respectively ($P < 0.05$). Degranulation capability of *in vitro* CD8$^+$ KIR2D$^+$ expanded cells was decreased compared to CD8$^+$ KIR2D- (22.6% for KIR2DL1/S1$^+$ and 24.6% KIR2DL2/L3/S2$^+$ and 46.5% for KIR-).
Conclusions: Our results are consistent with the *in vivo* observation of CD8$^+$ KIR2D$^+$ T cells expansion and its dependence on the liver recipient HLA-C allotype. Deeply analysis of these cells should be done in order to determinate its role in the alloresponse as possible regulatory cells.

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P0674

Human regulatory T cell subpopulation heterogeneity at single-cell level in homeostatic conditions and during graft-versus-host disease

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Purpose/Objective: CD4$^+$ FOXP3$^+$ regulatory T cells (Treg) are of critical importance for the control of immune homeostasis and the maintenance of tolerance. While Treg were initially considered a homogeneous and stable population, recent studies have identified multiple Treg subpopulations and their potential plasticity. Understanding of the heterogeneity and potential for lineage reprogramming of Treg to effector T cells with pro-inflammatory functions is key for advancing Treg therapy into the clinics.
Materials and methods: We have explored the heterogeneity and functional diversity of human Treg using multi-parameter single-cell analysis techniques in healthy donors and in patients developing acute graft-versus-host disease (aGVHD) after allogeneic hematopoietic stem cell transplantation (alloHSCT).
Results: Our single-cell analysis of the human Treg compartment revealed a pattern of highly variegated gene expression that has not been appreciated by the analysis of bulk populations or by flow cytometry. We found that IFN-γ or IL-17A-secreting CD4$^+$ FOXP3$^+$ Treg are characterized by an overlap of gene expression signatures of Th1 or Th17 cells and of Treg within the same cell. Of note, a substantial fraction of cytokine-producing Treg express *IKZF2* indicating thymic origin. Similar to FOXP3$^-$ CD4$^+$ effector T cells, the mosaic-like expression of transcription factors, signaling molecules and homing receptors can endow individual Treg with specific functions and migratory properties.

Analysis of Treg homeostasis in the severely inflammatory and lymphopenic environment in patients after alloHSCT at the time of engraftment did not provide evidence for lineage reprogramming of CD4$^+$ FOXP3$^+$ T cells. Although we observed a strongly skewed Treg compartment in patients developing aGVHD compared to the control group, the overall frequency of CD4$^+$ FOXP3$^+$ T cells and the suppressive activity of Treg isolated from aGVHD patients were preserved.
Conclusions: Our results show that the human Treg compartment displays a similar level of complexity and functional heterogeneity as conventional CD4$^+$ effector T cells. Furthermore, we found that altered homeostasis of functionally distinct Treg subpopulations, rather than the total number of Treg, is associated with acute GVHD.

P0675

ICOS-ICOSL regulates the generation and function of CD4$^+$ Treg in a CTLA-4 dependent manner

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Purpose/Objective: Inducible co-stimulator (ICOS, CD278) is a member of CD28/Cytotoxic T-lymphocyte Antigen-4 (CTLA-4, CD152) family and broadly expressed in activated CD4$^+$ T cells or induced regulatory CD4$^+$ T cells (CD4$^+$ Treg). The biology function of ICOS is mediated by the interaction between ICOS and its ligand (ICOSL, CD275). Here we investigate the role of ICOS-ICOSL in the generation and function of CD4$^+$ Treg and its underlying mechanisms.
Materials and methods: In our previous work, we established a cost-effective system to generate a novel human allo-antigen specific CD4$^+$ Treg by co-culturing their naïve precursors with allogeneic CD40-activated B cells *in vitro*. This induced CD4$^+$ Treg expressed high level of both ICOS and CTLA-4 on its surface. In this work, ICOS-Ig fusion protein and endocytosis inhibitors E64 and pepastatin A were applied to clarify the effects of ICOS-ICOSL and its correlation with the expression of CTLA-4.
Results: We found that blocking ICOS-ICOSL pathway impaired the induction and expansion of CD4$^+$ Treg activated by allogeneic CD40-activated B cells. Moreover, CD4$^+$ Treg induced with the supplement of ICOS-Ig exhibited decreased suppressive capacity on allo-responses. According to the molecular mechanisms, ICOS-ICOSL pathway signal was found to up-regulate the surface expression of CTLA-4 by both increasing the endocytosis and decreasing the exocytosis of CD4$^+$ Treg.

Conclusions: These data demonstrates the beneficial role of ICOS-ICOSL pathway in the generation of $CD4^+$ Treg through the interactions between co-stimulators (ICOS and CTLA-4) expressed in $CD4^+$ Treg.

P0676
Impaired homeostasis and function of circulating regulatory T cells in patients with coronary artery disease

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Purpose/Objective: Regulatory T cells (Tregs) are believed to be protective in atherosclerosis. Normally, numbers of naïve (*n*) Tregs decline with age while memory (m) Tregs are maintained or even increased in elderly. Studies of total Tregs in patients with coronary artery disease (CAD) have yielded contradictory results.

In the present study, we investigated whether CAD patients exhibited an impaired function and homeostasis of Tregs.

Materials and methods: We included 25 patients with stable CAD (all statin-treated) and 25 controls. Phenotypic analysis of Tregs using CD3, CD4, CD25, FoxP3, CTLA4 and Helios markers was performed *in vivo* by flow cytometry applying different gating strategies. Sorting of Tregs was performed using CD4, CD25, CD127 and CD45RA. Afterwards, nTregs and mTregs were analysed for FoxP3. In suppression assays, nTregs and mTregs were cocultured with T responder cells at ratios of 1:1, 1:4 and 1:10 for 18 h. Proliferation of responder cells was evaluated as well as cytokine secretion (IFN-γ, TNF, IL-2, IL-10) insupernatants.

Results: The frequencies of total Tregs *in vivo* ($CD3^+$ $CD4^+$ $CD25^{high}$, $CD3^+$ $CD4^+$ $CD25^{dim}$ and $CD3^+$ $CD4^{dim}CD25^{high}$) were similar in patients and controls. However, the proportion of nTregs ($CD4^+$ $CD25^{high}CD45RA^+$ or $CD4^+$ $CD25^+$ $CD127^-/^{low}CD45RA^+$) was significantly lower in patients, while the proportion of mTregs did not differ. The expression of activation markers CTLA-4 and Helios was higher on mTregs than nTregs but without any differences between patients and controls. However, the ability of both nTregs and mTregs to inhibit responder cell proliferation was significantly reduced in patients. The capacity of nTregs to inhibit the secretion of IFN-γ and TNF was also significantly decreased in patients and the capacity of both Treg fractions to induce secretion of IL-10 was significantly reduced in patients.

Conclusions: Using different gating strategies total Tregs was similar in patients and controls, however a significant reduction of nTregs in CAD patients was found. These results indicate an altered Treg homeostasis. Moreover, the function of both nTregs and mTreg was markedly impaired in patients. Although the clinical relevance remains to be elucidated, the data highlight the presence of immune dysfunction and immunosenescence in CAD patients.

P0677
Induction of $CD4^+$ regulatory T cells by soluble HLA-G isoforms

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Purpose/Objective: The non-classical HLA class I molecule HLA-G was initially shown to play a major role in feto-maternal tolerance. Since this discovery, it has been established that HLA-G is a tolerogenic molecule, which participates to the control of the immune response. HLA-G inhibits a wide array of immune cells and has long-term immune-modulatory effects since it can induce the generation of suppressor/regulatory cells. We recently demonstrated that tolerogenic DC, termed DC-10, promote the differentiation of Tr1 cells via the IL10-dependent membrane bound HLA-G1/ILT4 pathway. The role of membrane-bound HLA-G1 in promoting Tr1 cells *via* DC-10, raise the question whether soluble shed HLA-G1 or HLA-G5 can promote Tr1 cell differentiation.

Materials and methods: Human naïve $CD4^+$ T cells were stimulated via anti-CD3 mAbs cross-linked on artificial APC consisting in murine L-cells co-transfected with human CD32, CD80, and CD58 in the presence of shed sHLA-G1 or HLA-G5 alone or in combination with IL-10. As control, we used Th0 and Tr1 cells differentiated with artificial APC alone or in the presence of IL-10 and IFN-a, respectively.

Results: We showed that repetitive stimulation of human naïve $CD4^+$ T cells in the presence of shed HLA-G1 or HLA-G5, alone or in combination with IL-10, induced the differentiation of a population of $CD4^+$ T cells that are phenotypically different from both Th0 and Tr1 cells. T cells differentiated with soluble HLA-Gs secrete lower levels of IFN-g and IL-2 as compared to Th0 cells. Interestingly, HLA-G-induced T cells secrete low amounts of IL-10, which is slightly increased when IL-10 is present in culture, but never reach the levels produced by Tr1 cells. Despite the ability to proliferate upon polyclonal activation, HLA-G-induced T cells suppress the proliferation of autologous $CD4^+$ T cells *in vitro*.

Conclusions: We showed that activation of human $CD4^+$ T cells in the presence of both soluble shed HLA-G1 or HLA-G5, alone or in combination with IL-10, promotes the induction of a population of suppressor $CD4^+$ T cells, which are distinct from Tr1 cells.

P0678
Induction of IL-35 in human T cells upon co-stimulation via CD43 and PD-1

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Purpose/Objective: IL-35, an IL-12 family member, is a heterodimer of EBV induced gene 3 (EBI3) and p35. IL-35 has been characterised as an inhibitory cytokine and is extensively studied in mouse models. Recently, our group has shown the induction of IL-35 producing human regulatory T cells by human rhinovirus treated dendritic cells (R-DC) via up-regulation of B7H1 (CD274) and sialoadhesin (CD169). Thus, a combined set of co-stimulatory signals for T cells seems to be critical to induce IL-35. We therefore investigated the underlying molecular mechanisms involved on T cell side for the

induction of IL-35. In addition, we have also investigated the mechanism of action of IL-35 on human T cells.
Materials and methods: For the induction of IL-35, T cells isolated from human peripheral blood, were stimulated with a panel of plate-bound monoclonal antibodies (mAbs) against putative accessory T cell surface receptors.The IL-35 induction was analyzed by qPCR, intracellular staining and by using newly established ELISA. T cell cytokines were also measured. The inhibitory effect of R-DC induced IL-35 was also tested on T cells activated in presence or absence of antigen presenting cells (APC).
Results: Among the various combinations tested, T cells stimulated with plate-bound anti-PD-1 mAb (CD279) in combination with CD43mAb and CD3 mAb − OKT3 was identified to most potently induce IL-35, whereas the other T cell activation pathways failed to do so. T cells activated via $CD3^+$ $CD43^+$ PD-1 co-signaling showed a diminished proliferative response compared to cells stimulated via $CD3^+$ CD43. However, the T cell signature cytokine profile (IL-2, IL-4, IL-10, IL-17, IL-22, IFN-γ) remained unaltered upon stimulation via $CD3^+$ $CD43^+$ PD-1, compared to other activation protocols. Furthermore, the R-DC induced IL-35 exhibited the inhibitory effect only when T cells were activated in the presence of DCs.
Conclusions: Thus, suggesting that the presence of APC is rather essential for IL-35 mediated inhibition. From our data, human T cell stimulation via $CD3^+$ $CD43^+$ PD-1 seems to be a specific pathway to induce IL-35 production, that is not accompanied by re-organisation in the T cell signature cytokine profile and a novel pathway for the induction of immune inhibitory T cells.

P0679
Innate immune sensing by human regulatory T cells − role of TLR2 and relevance to autoimmunity in multiple sclerosis

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Purpose/Objective: We and others previously reported that TLR2 is more highly expressed on human $CD4^+$ $FOXP3^+$ $CD25^{hi}$ regulatory T cells (Tregs) and that activation of TLR1/2 heterodimers reduces Treg suppressive activity. We also found that TLR2 activation induces human Tregs to a Th-17-like phenotype and that similar to previour reports in the mouse, it can also induce human T helper precursors to Th17 differentiation. The objective of this study was to compare the modulation of $CD4^+$ Tregs by TLR2 agonists in people with and without multiple sclerosis (MS), an inflammatory demyelinating disease of the central nervous system in which defects of Treg function have been reported.
Materials and methods: For suppression assays, we used co-cultures of effector/responder T cells and Tregs purified using either CD25/CD127 or CD25/CD45RA sorting strategies (Miyara et al., Immunity 2009). These are APC-free cultures (Nyirenda et al., J Immunol 2011). Cells were cultured for 3−5 days in the presence or absence of TLR1/2 (Pam3Cys) or TLR2/6 agonists (Pam2Cys or FSL-1) and proliferation determined by incorporation of tritiated thymidine. For T-cell polarisation assays, CD4-enriched T cells were cultured in the presence or absence of a Th17 differentiation cocktail (IL-1, IL-16, TGF-beta, IL-23, anti-IFNgamma, and anti-IL-4) or the above mentioned TLR2 agonists. Intracellular expression of cytokine was measured by flow cytometry and cytokine secretion by ELISA or cytometric bead arrays.
Results: In a pilot study of 10 patients with MS and matched healthy controls, we found that the suppressive functions of $CD4^+$ $CD25^{hi}CD127^{neg}FOXP3^+$ Tregs as well as $CD45RA^+FOXP3^{lo}$ (naïve Tregs) and $CD45RA^-FOXP3^{hi}$ (effector Tregs) Treg subsets from MS patients were more susceptible to TLR2-induced reduction of suppressive function and expression of Th17 cytokines than those derived from healthy controls. Blocking IL-6 with a neutralising Ab reversed TLR2-induced loss of suppressive function and conversely, stimulation of $CD4^+$ T cells with Pam3Cys enhanced the expression of IL-6 and pSTAT3 in cultured T cells, pointing to the importance of IL-6 signalling in mediating the effects of TLR2 activation.
Conclusions: These data suggest that infections that are able to stimulate TLR2 *in vivo* may modulate the Treg/Th17 balance in MS patients, facilitating the elimination of infectious agents at the possible cost of increased risk of autoimmune reactions. A degree of flexibility in recognition of pathogen- or danger-associated molecular patterns by TLR2 suggests that not only bacterial, but also viral and self structures may act as agonists *in vivo*.

P0680
Interferon-alpha plays complex roles in human regulatory T cell deprogramming into Th1-like cells

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Purpose/Objective: Regulatory T cells (Treg) are classically viewed as immune suppressive cells tipping the balance between host defence from pathogens and prevention of chronic inflammation and autoimmunity. Recent data indicate that Treg can dynamically adapt to different contexts and even dampen their suppressive program while acquiring the competence for unexpected inflammatory functions. The cytokine context may dictate the Treg fate decision between stability and deprogramming. Very little is known about the effects of interferon (IFN)-alpha on Treg. Indeed this cytokine, beyond a well-known antiviral activity and a pathogenic role in some autoimmune diseases, may display specific immunomodulatory or immunostimulatory properties in different settings. The purpose of this study was to characterize the functional changes induced in Treg upon exposure to IFN-alpha.
Materials and methods: Treg obtained from healthy donors were cultured with recombinant IFN-alpha under neutral (IL-2) or Th1-prone (IL-2^+ IL-12) conditions and Treg functions were tested by flow cytometry in terms of expansion, suppression, Tbet and IFN-gamma expression. To evaluate the effects of Treg exposure to IFN-alpha *in vivo*, Treg were similarly analysed by flow cytometry in patients with chronic HCV infection undergoing peginterferon/ribavirin therapy, before and 2 days after the starting of therapy.
Results: *in vitro*, in neutral culture conditions, IFN-alpha slightly increased Tbet and IFN-gamma expression by Treg; in the Th1-prone condition, the addition of IFN-alpha further enhanced IL-12-driven IFN-gamma production while slightly reducing Tbet upregulation, suggesting a complex role for IFN-alpha in supporting Treg deprogramming into Th1-like cells. IFN-alpha also exerted an anti-proliferative effect against both conventional T cells (Tconv) and Treg, without affecting anyway the Treg-intrinsic suppressive function in Tconv-Treg cocultures under neutral conditions. In the majority of HCV patients treated with peginterferon/ribavirin, we could observe a decreased Treg frequency and an enhanced IFN-gamma production by both Tconv and Treg after treatment. IFN-gamma secretion by the two cell subsets showed a linear correlation, suggesting that Tconv-derived IFN-gamma may play a role in Treg diversion into Th1-like cells.
Conclusions: Our data indicate that, under Th1 conditions, IFN-alpha may amplify Th1-type inflammation by inhibiting Treg expansion and fostering Treg deprogramming into Th1-like cells.

P0681

Modulation of Treg-mediated supression by iNKT cells in T1D

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Purpose/Objective: Type 1 Diabetes (T1D) is an organ-specific autoimmune disease mediated by the destruction of insulin-producing beta cells by T cells. T regulatory cells (Tregs) and invariant natural killer T cells (iNKT) are important to control pathogenic autoreactivity. Results from our group showed that both cell types were present at the diabetic pancreas although Tregs were preferentially located inside the islets while iNKTs migrated to the islets at late stages of the disease. An active cross-talk between Tregs and iNKTs at the target organ could result on a better regulation of autoagressive T cells. Thus, our aim was to study their functional interaction in T1D patients.

Materials and methods: iNKT and Treg cells were selected from healthy donors and T1D patients PBMCs based on the expression of the TCR α-chain Vα24Jα18 or $CD4^+$ $CD25^{++}$ and expanded *in vitro* with $CD1d^+$ APCs pulsed with αGalactosylCeramide or αCD3, respectively. We analyzed the capacity of the expanded iNKTs cells to modify the suppressor effect of Tregs in a proliferation assay in the presence of increasing numbers of iNKTs. We determined if the interaction needed cell-cell contact or cytokine secretion using a transwell system.

Results: The results showed that Treg cells suppressed Teff cell proliferation by limiting their IL2 production in a cell-cell contact-dependent manner. On the other hand, iNKTs increased up to a 40% the Teff proliferation supression by Tregs. This effect so far appears to be independent of cell-cell contact between INKTs and Tregs. The analysis of cytokine secretion demonstrated that iNKTs contributed to the supression of Teff cells maintaining a low IL-2 secretion rate. At the same time, iNKTs enhanced the secretion of other effector cytokines by Teff. This was confirmed by abrogation of cytokine secretion by the use of irradiated iNKTs or Teff. The possible secretion of soluble inhibiting factors by iNKTs that could enhance the Treg suppression is currently under investigation.

Conclusions: iNKT cells have an adjuvant effect on Tregsfunction as they increase their capacity to inhibit Teff cell proliferation *in vitro*. The co-localization of these two cell populations in the diabetic pancreas suggests that this effect could be taking place *in vivo*. This adjuvant effect could represent a tool to overcome the proposed resistance to suppression by Tregs of diabetic Teff cells.

P0682

Ontogeny, phenotype and functional properties of human $CD39^+$ regulatory T cells

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Purpose/Objective: CD39 and CD73 are ectoenzymes that act in concert to hydrolyze extracellular ATP to generate adenosine, a molecule with anti-inflammatory properties. While murine Treg express both CD39 and CD73, and CD39-deficient animals show impaired regulatory function, the picture in humans is more complicated. $CD39^+$ Tregs can uniquely constrain Th17 cell responses, and are impaired in patients with multiple sclerosis. However, it remains the question whether the expression of CD39 is intrinsic to a particular subset of Tregs or if CD39 is transiently upregulated in activated Tregs.

Materials and methods: We used multiparameter flow cytometry to analyze the phenotpye and functional properties of the $CD39^+$ and CD39- Treg subsets in the thymus, cord blood and peripheral blood from children and adult donors. Using cellular dyes, we labeled the $CD39^+$ and CD39- subsets of Tregs in order to track them after activation, and performed proliferation assays to determine their respective suppressive capacity.

Results: Phenotypically, $CD39^+$ Tregs express higher levels of FOXP3, display markers characteristic of memory cells, and express chemokine receptors that can guide them to sites of inflammation. However, the expression of the activation marker CD69 is similar in the two subsets, and $CD39^+$ Tregs express lower levels of CD26 and CD38 compared to CD39- Tregs. Upon polyclonal activation, CD39- Tregs upregulate CD39 modestly, never reaching the levels of the $CD39^+$ Tregs. Interestingly, only $CD39^+$ Tregs upregulate CD73, which makes them more efficient at producing adenosine. Both subsets are suppressive, although $CD39^+$ Tregs are more efficient than their CD39- counterparts. In patients with juvenile rheumatoid arthritis, $CD39^+$ Treg are highly elevated in the synovial fluid, probably reflecting an attempt to control chronic inflammation. Regarding their origin, $CD39^+$ Tregs are already found in the thymus, and CD39 is not only expressed in a subset of the single positive $CD4^+$ $CD25^+$ thymocytes, but also by some double positive precursors. Cord blood cells also contain $CD39^+$ Tregs, although at lower levels that later in life, and $CD39^+$ Tregs were found to increase with age. Of note, in peripheral blood from adults both the $CD39^+$ and the CD39- subsets contain $CD31^+$ cells, indicating recent egress from the thymus.

Conclusions: Altogether, our results support the notion that CD39 does not reflect an activated status of Treg cells, but rather a subset that also originates in the thymus, displays specific migratory properties and is more efficient at suppression.

P0684

p27kip1 inhibits systemic autoimmunity through the control of regulatory T cell activity and differentiation

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Purpose/Objective: Despite the importance of regulatory T cells (Tregs) in the maintenance of immunological tolerance, little information is still available about the molecular mechanisms that regulate the number and activity of these cells *in vivo*. Using different experimental approaches, other authors showed that Bcl-xl affected the development and activity of Tregs; however, the precise molecular mechanism by which these anti-apoptotic factors influenced Tregs was not elucidated in such studies. Since the cell cycle inhibitor $p27^{kip1}$ (p27) was involved in T-cell anergy, we also explored here its role in both Treg processes.

Materials and methods: (DBA/1 × B6) F1 Tg mice overexpressing human Bcl-2 in T cells and (DBA/1 × B6) F1 $p27^{+/-}$ were immunized with 150 μg of collagen bovine type II emulsified with complet Freund's adjuvant for the induction of Collagen-Induced Arthritis.

Depletion of Tregs was performed using an anti-CD25 mAb

For supression cocultures, $CD4^+$ CD25- effector T cells and $CD4^+$ $CD25^+$ regulatory T cells were purified by cell sorting and stimulated with anti-CD3 and antigen presenting cells under different Treg/Teff ratios.

In the differentiation cultures, $CD4^+$ $CD25^-CD62L^+CD44^-$ naïve T cells furified by cell sorting were cultured with anti-CD3/CD28 under Treg inducing conditions (TGFβ and IL-2).

Results: First, we demonstrated here that Tg mice overexpressing human BCL2 in T cells (BCL2-TgT mice) were protected against collagen type II-induced arthritis (CIA) by a Treg-dependent mechanism. In association with this protection, the overexpression of BCL2 in T cells enhanced the differentiation and activity of Tregs. Both BCL2 effects were independent of its anti-apoptotic activity but dependent on its capacity to induce the expression of the cell cycle inhibitor p27 that enhanced the strength of TGFb-signalling in T cells. Accordingly, the modulation of p27 expression in BCL2-TgT mice promoted CIA. In addition, p27 deficiency in aged B6 mice reduced the number and activity of Tregs and induced the development of autoimmunity.
Conclusions: Our results pointed to p27 as a critical regulator of Treg differentiation and function through the positive modulation of TGFb-signalling strength in T cells.

P0686
Potential induction of regulatory T cells by 5-Azacytidine and EGCG from non-regulatory T cells

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Purpose/Objective: Regulatory T cells (Treg cells) play a pivotal role in establishing a peripheral immune tolerance. A deficit of Treg cells is supposed to play an essential role in Graft versus Host disease after bone marrow transplantation. Adoptive transfer of Treg cells is hoped to inhibit development or improve the course of disease. One approach is the *in vitro* induction of Treg cells by demethylating agents, as human Treg cells differ from non-regulatory T cells by protein expression as well as epigenetic differences in DNA methylation. The two DNA methyltransferase (DNMT) inhibitors, 5-Azacytidine (5-AZA), which is used for the treatment of myelodysplastic syndrome and epigallocatechin-3-gallate (EGCG), which is a main component of green tea, were analyzed for their potential in inducing a Treg cell phenotype on DNA-, RNA- and protein-level.
Materials and methods: Jurkat T cells and FACS-sorted human $CD4^+$ $CD25^-$ cells were analyzed after cultivation with 5-AZA or EGCG. DNA-methylation, FOXP3 protein expression by flow cytometry and FOXP3- and GARP-RNA-expression by real-time PCR after 4 and 7 days of cultivation were analyzed.
Results: 5-AZA induces a strong global DNA-demethylation and a FOXP3- and GARP-expression on protein as well as RNA-level in Jurkat and $CD4^+$ $CD25^-$ T cells. The potential of EGCG in global DNA-demethylation is similar to 5-AZA, but EGCG has only a very low potency of inducing FOXP3- expression in Jurkat and sorted $CD4^+$ $CD25^-$ T cells, while it was not able to induce GARP-expression in Jurkat and sorted $CD4^+$ $CD25^-$ T cells.
Conclusions: 5-AZA seems to be more suitable for induction of Treg cells than EGCG. We are currently analysing further phenotypic and also functional properties of the induced cells.

P0687
Quantification of regulatory T cells in septic patients by real-time PCR–based methylation assay and flow cytometry

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Purpose/Objective: During sepsis, a relative increase of regulatory T cells (Treg cells) has been reported. Its persistence is associated with a poor prognosis, lymphocyte anergy and immunoparalysis. As an exact quantification of human Treg cells by protein expression is currently unfeasible due to a lack of specific Treg cell molecules, a more precise and reliable method of quantifying Treg cells in septic patients is needed.
Materials and methods: We established a single-tube real-time PCR-based methylation assay for quantification of human Treg cells. This assay takes advantage of a Treg cell specific pattern within *FOXP3-TSDR*. We analyzed DNA-methylation of $CD4^+$ -isolated cells in blood samples from 30 septic patients and 30 healthy subjects in comparison to flow cytometric quantitation of $CD4^+$ $CD25^{hi}CD127^{low}$ T cells indicative of Treg cells.
Results: Both real-time PCR and flow cytometry demonstrated that the ratio of Treg cells to all $CD4^+$ cells was significantly higher in septic patients than in healthy subjects. The results yielded by the two methods were clearly positively correlated in healthy individuals, while they were only weakly positively correlated in septic patients.
Conclusions: The described methylation-sensitive real-time PCR assay of *FOXP3-TSDR* precisely quantifies natural Treg cells in septic patients by using the most specific feature of these cells known thus far. It allows for more reliable immune monitoring of Treg cells in septic patients. Due to the known altering protein expression phenotype of $CD4^+$ T cells after activation, results obtained by flow cytometry especially in the collective of septic patients should be considered with caution.

P0688
Rarity of human t helper 17 cells is due to retinoic acid orphan receptor-dependent mechanisms that limit their expansion

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Purpose/Objective: We investigate the reason why $CD4(^+)$ T helper 17 (Th17) cells, despite their well-known pathogenic role in chronic inflammatory disorders, are very rare in the inflammatory sites.
Materials and methods: The ability of Th17 and Th1 human T cell clones to respond upon anti-CD3-CD28 or PMA plus ionomycin stimulation and their signaling activation pathway was investigated.
Results: We demonstrate that human Th17 cells exhibit low ability to proliferate and to produce the T cell growth factor interleukin-2 (IL-2), in response to combined CD3 and CD28 stimulation. This was due to the upregulated expression of IL-4-induced gene 1 (IL4I1) mRNA, a secreted L-phenylalanine oxidase, which associated with a decrease in CD3ζ chain expression and consequent abnormalities in the molecular pathway that allows IL-2 production and cell proliferation. High IL4I1 mRNA expression was detectable in Th17 cell precursors and was strictly dependent on Th17 cell master gene, the retinoid acid related orphan receptor (RORC). Th17 cells also exhibited RORC-dependent CD28 hyperexpression and the ability to produce IL-17A after CD28 stimulation without CD3 triggering.
Conclusions: Our findings suggest that the rarity of human Th17 cells in inflamed tissues results from RORC-dependent mechanisms limiting their expansion.

P0690
Regulatory T cells induced by mucosal B cells alleviate allergic airway hypersensitivity

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Purpose/Objective: Asthma is one of the most common chronic airway inflammatory diseases. Induction of immunologic tolerance via

mucosa has been used for treating allergic diseases. B cells, the major cell population in Peyer's patches, have been shown to induce the development of Treg cells. This study aimed to investigate the role of B cells in Peyer's patches to tolerance induction and on Treg cell functions.

Materials and methods: *In vitro* suppressive assay and ELISA were used to evaluate the function of T cells stimulated by Peyer's patches B cells (Treg-of-B cells), the therapeutic potential of which was evaluated by an animal model of airway inflammation.

Results: Treg-of-B cells were found to exert suppressive function on T cell proliferation. Antigen-loaded B cells isolated from Peyer's patches were more tolerogenic and had the potential to generate more suppressive Treg-of-B cells via IL-10 production and cell-cell contract. Treg-of-B cells expressed CTLA4, ICOS, OX40, PD-1, and TNFRII and produced lower IL-2 and higher IL-10. In the murine model of asthma, an adoptive transfer of Treg-of-B cells pre- or post- immunization could sufficiently suppress Th2 cytokine production and eosinophil infiltration, and alleviate asthmatic symptoms.

Conclusions: B cells isolated from gut-associated lymphoid tissues, GALT, can generate regulatory T cells that may be important in oral tolerance and be applicable for alleviating allergic symptoms.

P0691

Role of the innate immune system in CD8$^+$ T regulatory T cell differentiation

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Purpose/Objective: Regulation of the immune system plays an important role in maintaining homeostasis in the human body. It«s role is of particular importance in autoimmune disorders, organ transplanting and the body's defences against pathogens and cancer. Recently growing interest is upon the role of CD8$^+$ T regulatory cells (T_{regs}) in this context. The aim of this study was to evaluate the role of the innate immune system in CD8$^+$ T_{Regs} differentiation and its regulatory capacity.

Materials and methods: naïve human CD8$^+$ T-cells, defined as CD25$^-$ CD45RA, were isolated and differentiated for five days in anti-CD3 coated plates with CD28 (1 μg/ml), IL-2 (200 IU) and TGF-β1 (10 μg/ml) with or without IL-1β (2.5 μg/ml) or TNF-α (10 μg/ml). To evaluate their suppressive function, cells were co-cultured with CFSE stained PBMC«s and Epstein-Barr infected B-cells charged with superantigens (EBB). The CD8$^+$ T_{Regs} differentiation of naïve CD8$^+$ T-cells was also tested in culture with allogeneic mature monocyte derived dendritic cells (mDC) with or without IL-2 and TGF-β1. Cells were analysed by flow cytometry to access their phenotype. CD8$^+$ T_{Regs} phenotype was defined as CD8$^+$ CD127$^-$ CD25^{high} FoxP3$^+$ (CD8$^+$ $_iT_{Regs}$).

Results: Exogenously added IL-1β and TNF-α did not suppress the differentiation of CD8$^+$ $_iT_{Regs}$ (%differentiated without IL-1β or TNF-α = 19.4% v.s. with IL-1β = 18.0% v.s. with TNF-α = 17.15%; P = n.s.). However the co-addition of IL-1β prevented $_iT_{Regs}$ suppressive function (%proliferating, $_iT_{Regs}$ 15.2% v.s. $_iT_{Regs}$ with IL-1β 22.3%; $P < 0.05$). Activation of naïve CD8$^+$ T-cells with allogeneic mDC resulted in a significant differentiation of CD8$^+$ $_iT_{Regs}$ (9.5%, $P < 0.03$). In addition such differentiation was enhanced by the exogenous administration of IL-2 and TGF-β1 (% differentiated without IL-2/TGF-β19.5% v.s. with 15.9%). Finally mDC expression of CD80 and CD86 was significantly downregulated following CD8$^+$ $_iT_{regs}$ expansion.

Conclusions: The study indicates that IL-1β and TNF-α do not affect CD8$^+$ $_iT_{Regs}$ differentiation. However it suggests that IL-1β may infer with its functionality. In addition, following stimulation with mDC significant proportion of nonresponding CD8$^+$ T-cells differentiate into $_iT_{Regs.}$ This may enhance our understanding of the interplay of innate immunity in autoimmunity.

P0692

Sequential expression of CD39 regulates late developmental Th17 plasticity imparting a regulatory cell phenotype

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Purpose/Objective: CD39 is an immune cell phenotype marker that exhibits ectonucleotidase activity, converting extracellular nucleotides to nucleosides. Although CD39 has been associated with Treg, the ectoenzyme is also expressed by a subpopulation of memory T-cells (CD4^{mem}) with effector functions. We postulate that CD39 imparts plasticity to effector T helper type 17 (Th17) as well as co-ordinating Treg cellular programs of differentiation.

Materials and methods: Experiments were performed using peripheral blood mononuclear cells obtained from healthy blood donors. Sorted CD4$^+$ CD45RO$^+$ memory cells (CD4^{mem}) were exposed to 1) IL6$^+$ IL1β^+rTGFβ or 2) IL6$^+$ IL1β^+IL23 or 3) IL6$^+$ IL1β^+rTGF-β^+IL23 to induce Th17 polarization. Cells at Th17 stage were then treated with high dose IL2$^+$ anti-CD3/anti-CD28 to favour Treg differentiation and then re-exposed to Th17 differentiating conditions to induce putative reverse or suppressive Th17 (rev/regTh17) cells. Impacts of purinergic mediators on cell effector phenotype and functions were assessed.

Results: CD4^{mem} could be differentiated sequentially to Th17, Treg and rev/regTh17. In contrast to the inflammatory properties associated with prototypic Th17 cells, rev/regTh17 exhibited a suppressive phenotype (i.e. CD39^{high}, CD73^{high}, FOXP3$^+$) and were able to control CD4$^+$ CD25$^-$ cell proliferation and pro-inflammatory cytokine (IFNγ, IL17) production. At variance with Treg that following exposure to adenosine up-regulated CD39, CD73 and FOXP3 and became more suppressive, rev/regTh17 remained resistant to adenosine.

Conclusions: Differential levels of expression of CD39 designate early Th17 cells from later Treg/revTh17 cell plasticity. The potential for Treg to revert to the inflammatory Th17 phenotype is mitigated by expression of CD39, as indicated by enhancements of suppressive function *in vitro*.

P0693

Shifting gears: Immune system parameters of middle-aged marathon runners versus sedentary controls

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Purpose/Objective: To investigate the effect of strenuous exercise on the immune system and whether habitual long term exercise can actually preserve an immunological capacity compared to sedentary lifestyle, we examined lymphocyte subsets, cytokine levels and biochemical data of middle age marathon runners versus sedentary controls.

Materials and methods: Blood samples were collected from controls only once, whereas from marathon runners at three time points: within 1 h after the race, and seven days before and after the race. Routine complete blood counts were determined using an automatic blood counter. Major cytokines and cortisol levels were measured by enzyme-linked immunosorbent assays and peripheral blood lymphocyte subsets

were determined by two-color flow cytometry. Cytokine mRNA levels were measured by polymerase chain reaction (PCR).
Results: The race induced peripheral leukocytosis, attributed to neutrophilia. Plasma CPK and cortisol concentrations also increased significantly after the race. $CD4^+$ memory cells, total $CD8^+$ cells, $CD8^+$ naïve cells, $CD8^+$ memory cells, activated $CD8^+$ cells, NK cells, B cells and B1α cells were decreased after the race, whereas T regulatory cells ($CD4^+$ $CD25^+$ $FoxP3^+$) count was elevated significantly. Serum IL-1b, IL-6, IL-4, IL-10 and TGF-β1 levels were raised significantly post race, similar to mRNA levels measurements. One week later cytokine concentrations had dropped to original levels before the race, except for IL-2R, IL-4 and TGF-β1 which remained high. Compared to sedentary controls, B1α cells, activated $CD8^+$ cells and IL-2R concentration were at higher levels, while $CD4^+$ memory cells, activated $CD4^+$ cells and TGF-β1 levels were significantly lower.
Conclusions: Strenuous exercise results in a pro-inflammatory phenotype, as seen by the recruited cells and the secreted cytokines. This leads to suppression of host's defense and could predispose to autoimmunity. However, due to chronic regular exercise, athletes have developed regulatory mechanisms which balance effectively every polarization of the immune response and no cumulative effect of oxidative stress on cytokine balance seems to occur.

P0694

Study on the molecular mechanisms involved in the generation of regulatory T cells induced by B lymphocytes

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Purpose/Objective: B lymphocytes have multiple functions in the immune system. In addition to the role in the humoral immunity, some recent studies have reported that B cells preferentially expand $Foxp3^+$ $CD4^+$ regulatory T cells (Tregs). It is well-known that the transcription factor Foxp3 confers the regulatory function of naturally occurring Tregs. Based on previous researches, it is speculated that B cells might play an important role to induce functional Tregs. However, efficient generation of Tregs is still an ongoing work.
Materials and methods: naïve splenic B and $CD4^+$ CD25-T cells cells from female Balb/c mice co-cultured together with anti-CD3/ anti-CD28 antibodies. After 3 days, the T cells were collected and measured for the suppressive ability by [3H] thymidine incorporation. Analysis of cell surface marker was carried out using a FACScan instrument and the cytokine profiles were measured by ELISA.
Results: The preliminary results showed while co-culture of naïve splenic B cells with $CD4^+$ CD25-T cells, the T cells converted into $CD4^+$ $CD25^+$ T cells (Treg-of-B) which could suppress proliferative response of $CD4^+$ CD25- responder T cells. The phenotypic analysis demonstrated that Treg-of-B cells expressed high level of ICOS and GITR but not Foxp3. The supernatant of T-B co-culture showed the production of IL-10, IL-4 and IL-2. The cytokine profile evolved over time, following second rounds of restimulaion with naïve fresh B cells, and resulted in the increased IL-10 production.
Conclusions: These results suggested the splenic naïve B cells can efficiently induce functional IL-10-producing Tregs *in vitro*. Some surface makers are similar to natural Tregs although the expression of Foxp3 is not a prerequisite for Treg-of-B activity. Thus, Treg-of-B cells can be exploited for the control of peripheral immunity or might be involved in the prevention of autoimmunity.

P0695

Targeted delivery of neural autoantigen to liver sinusoidal endothelial cells protects from experimental autoimmune encephalomyelitis

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Purpose/Objective: Previously, we demonstrated that delivery of neural autoantigen to the liver by gene transfer induced TGF-β dependent conversion of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Treg) from conventional $CD4^+$ CD25- T cells and protected mice from experimental autoimmune encephalomyelitis (EAE) (LŸth S et al., J Clin Invest 2008;118: 3403−10). However, the relevant liver cell types involved in hepatic Treg generation remained undefined.

Therefore, we aimed 1) to identify the liver cell type that can induce Treg conversion, and 2) to explore whether targeted delivery of autoantigen peptides to these Treg-inducing liver cells may serve the treatment of autoimmune disease.
Materials and methods: To assess the capacity of liver sinusoidal endothelial cells (LSEC) to induce Treg from $CD4^+$ CD25-Foxp3-non-Treg cells, we stimulated myelin basic protein (MBP)-specific non-Treg from tg4 × foxp3-gfp mice on primary LSEC or, as control, on splenic dendritic cells (DC, 5×10^4) in the presence of MBP-peptide (5 ng/ml). The suppressive activity of LSEC-induced Treg was assessed by transfer of 10^5 LSEC-stimulatedT cells to MBP-immunised mice one day after EAE induction. Alternatively, MBP peptides were delivered to LSEC of B10.PL mice *in vivo*, one day after EAE induction, by using a novel nanoparticle carrier, which selectively targets LSEC.
Results: Stimulation of non-Treg by LSEC, but not by DC efficiently induced antigen-specific $CD4^+$ $CD25^+$ $Foxp3^+$ Treg in the presence of exogenous TGF-β (30.4% versus 5.9%; $P = 0.0022$). Moreover, transfer of LSEC-stimulated $CD4^+$ T cells to MBP-immunised mice conferred almost complete protection from EAE for more than 20 days (EAE score 0.6 versus 3.1; $P = 0.0006$), after which recipient mice developed only mild disease symptoms. Importantly, the targeted delivery of MBP-peptides to LSEC *in vivo,* using nanoparticles that specifically target LSEC, efficiently induced complete and lasting protection from EAE (0.6 versus 4.0; $P = 0.0008$); in contrast, application of unloaded nanoparticles or free MBP-peptide did not alter EAE severity(EAE score 4.2).
Conclusions: We identified LSEC as a liver cell type capable of inducing TGF-β dependent antigen-specific Treg conversion. Therefore, nanoparticle-based delivery of autoantigen to LSEC may be a treatment option for autoimmune diseases.

P0696

The dependence on NFAT levels discriminates conventional T cells from Foxp3$^+$ regulatory T cells

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Purpose/Objective: Several lines of evidence suggest NFAT to control regulatory T cells: thymus-derived nTregs depend on calcium signals, the *Foxp3* gene harbors several NFAT binding sites and the Foxp3 protein interacts with NFAT. Therefore, we investigated the impact of NFAT on Foxp3 expression.
Materials and methods: *In vitro* and *in vivo* generated iTregs as well as nTregs from different NFAT deficient mice were analysed by FACS, immunofluorescence, immunoblot, PCR, ELISA, ChIP assays, and microarrays as well as adoptive transfer colitis and a skin transplant model.
Results: The generation of iTregs by TGFβ was highly dependent on NFAT expression as the ability of CD4$^+$ T cells to differentiate into iTregs diminished markedly with the number of NFAT family members missing. It can be concluded that the expression of Foxp3 in TGFβ-induced iTregs depends on the threshold value of NFAT rather than on an individual member present. This is specific for iTreg development, since frequency of nTregs remained unaltered in mice lacking NFAT1, NFAT2 or NFAT4 alone or in combination. Different from expectation however, the function of both nTregs and iTregs was independent on robust NFAT levels, reflected by less nuclear NFAT in nTregs and iTregs. Accordingly, absence of one or two NFAT members did not alter suppressor activity *in vitro* or during colitis and transplantation *in vivo*.
Conclusions: This scenario emphasizes an inhibition of high NFAT activity as treatment for autoimmune diseases and in transplantation, selectively targeting the proinflammatory conventional T cells, while keeping Tregs functional.

P0697

The differentiation and suppressive function of CD8$^+$ T regulatory cells

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Purpose/Objective: CD8$^+$ T regulatory cells (CD8$^+$ T_{Regs}) display an immune-regulatory activity and play essential role as effector T-cells in several immune-pathologic manifestations such as autoimmune diseases (RA, SLE, MS), transplantation and protection of the host against infectious diseases and cancer. Therefore, they have a great potential as therapeutic targets and further research on surface markers, mRNA expression and cytokine secretion is needed to understand the cellular and molecular mechanisms underlying their mediated immune-suppressant. Our main objectives are to investigate the existence of induced CD8$^+$ T_{Regs} (CD8$^+$ iT_{Regs}) by inducing naïve CD8$^+$ CD25$^-$ T-cells *ex vivo* and confirm their suppressive activity.
Materials and methods: CD8$^+$ CD25$^-$CD45RA$^+$ were isolated from PBMCs and cultured in anti-CD3 coated plates with or without IL-2, TGF-β1 or CD28. Their phenotypic properties were analysed after 5 days using flow-cytometry. Furthermore, their suppressive activity was investigated by co-culturing developed CD8$^+$ iT_{Regs} with CFSE (carboxyfluorescein succinimidyl ester) labelled PBMCs and Epstein-Barr infected B-cells charged with superantigens (EBB).
Results: The *ex vivo* differentiation of human naïve CD8$^+$ T-cells into CD127-/CD8$^+$ /CD25^{high}/FoxP3^{high} iT_{Regs} was evaluated. It was demonstrated that it was dependent upon the presence of both TGF-β1 and IL-2 (%differentiation; CD3/CD28/IL-2: 4.2%, CD3/CD28/IL-2/TGF-β1: 14.4%; $P < 0.02$). Furthermore, it was found that the differentiation was dose dependent regarding exogenously added IL-2 ($P < 0.05$). Interestingly co-stimulation through CD28 did not have any additive effect upon CD8$^+$ iT_{Regs} differentiation. Additionally, the suppressive function of CD8$^+$ iT_{Regs} was confirmed to inhibit the proliferation of CD8$^+$ and CD4$^+$ T-cells.
Conclusions: The study presents the existence of CD8$^+$ T_{Regs} in human. Whereas their differentiation can be mediated *ex vivo* it increases our hope that it can be exploited as therapeutic treatment in T-cell mediated auto-immune diseases.

P0698

The effects of proinflammatory cytokines on the differentiation and function of human CD$_4^+$ induced regulatory T cells

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Purpose/Objective: Regulatory CD4$^+$ T cells (T_{Regs}) are one of the key elements of peripheral tolerance. T_{Regs} are divided into two major classes; central natural T_{Regs} (nT_{Regs}) and peripherally induced T_{Regs} (iT_{Regs}) which are phenotypically indistinguishable but may differ particularly regarding their differentiation and function. It has been revealed that the innate immune system plays a significant role in the immunopathology of autoimmunity. However, its part in differentiation and function of human CD4$^+$ iT_{Regs} is still unclear. The aim of this study was to evaluate the effects of proinflammatory cytokines on the development and function of CD4$^+$ iT_{Regs}.
Materials and methods: Naive human T cells (CD4$^+$ CD25$^-$) were isolated from PBMC's and stimulated with 1 mg/L anti-CD3 and cultured in the presence of IL-2 (100 IU) and TGF-β1 (10 ng/ml) with and with/without IL-1β and TNFα for 5 days. The phenotype of iT_{Regs} was defined (CD4$^+$ /CD127$^-$/CD25^{hi}/Foxp3$^+$) and analysed by flow cytometry. The iT_{Regs} were then cocultured with CFSE labelled PBMC's and Epstein-Barr infected B cells pulsed with superantigens. The proliferation was assessed by flow cytometry. Statistical analysis was performed after analysis with Modfit.
Results: The highest fraction of *ex vivo* differentiated CD4$^+$ iT_{Regs} from naïve human CD4$^+$ T cells was found to be dependent upon the presence of both IL-2 and TGF-β1 ($P < 0.05$). IL-1β and TNFα significantly inhibited the differentiation of iT_{Regs} (% inhibition; TNFα = 68,3% versus IL-1β = 73,5%; $P < 0.05$). The suppressive function of *ex vivo* induced CD4$^+$ iT_{Regs} correlated positively with their increasing numbers (% suppression; 1:1 = 49.75 versus 1:32 = 18.12; $P < 0.05$). IL-1β and TNFα were both found to significantly inhibit the effector function of CD4$^+$ iT_{Regs} ($P < 0.05$). Finally, CD4$^+$ iT_{Regs} significantly suppressed the proliferation of both CD4$^+$ and CD8$^+$ human T cells in our system.
Conclusions: Our study demonstrates that *ex vivo* differentiation of human iT_{Regs} results in a highly functional CD4$^+$ iT_{Regs}. It is also clear that the proinflammatory cytokines of the innate immune system have a significant negative effect upon their differentiation and function. These findings may reflect possible immunopathogenic mechanisms behind autoimmune diseases driven through overactive innate immune responses.

P0699

The role of regulatory T cells during mCMV infections

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Purpose/Objective: *Cytomegaloviruses* (*CMV*) set up latent infections in their hosts. In immunocompetent humans infection with *CMV* leads to a mild illness, but in immunocompromised patients primary infection as well as reactivated persistent virus cause life threatening diseases.

There is a growing body of evidence that regulatory T cells (Tregs) contribute to the establishment of persistent infection of different pathogens. Therefore we are interested in the impact of Tregs in the establishment of latency during *CMV* infections. Moreover, it has previously been shown that the immunosuppressive cytokine IL-10 has an impact on the outcome of murine *CMV* (*mCMV*) infections.

Materials and methods: In this study we used the well established mouse model for *CMV* infections. BALB/c mice were infected with *mCMV* Smith or *mCMV-Δm157-luc* strain. The frequency of Foxp3$^+$ Tregs and the mRNA profile of CD4$^+$ T cells in the course of infection were analyzed.

To shed light on the role of T cell derived IL-10 we infected *IL-10*$^{fL/fL}$ × CD4-Cre$^+$ mice, lacking IL-10 in T cells. FACS analyses on the activation status and cytokine production of T cells during *mCMV* infection were performed. For Foxp3$^+$ Treg depletion experiments we made use of the DEREG mouse model.

Results: We found that the frequency of Tregs does not alter during *mCMV* infection but isolated CD4$^+$ T cells showed increased levels of IL-10 mRNA.

In first experiments we could show that the T cell activation status was increased in *mCMV* infected *IL-10*$^{fL/fL}$ × CD4-Cre$^+$ mice in contrast to wild-type mice, determined by the downregulation of CD62L in addition to an increase in IFNγ and GzmB production. Similar results were obtained from Foxp3$^+$ Treg-depleted *mCMV* infected DEREG mice.

Conclusions: Our preliminary data suggest that Foxp3$^+$ regulatory T cells together with T cell derived IL-10 have an impact on the outcome of *mCMV* infections.

P0700

the role of the casein kinase 2 in regulatory t cell mediated suppression

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Purpose/Objective: During the last 20 years CD4$^+$ CD25$^+$ FoxP3$^+$ regulatory T cells (Tregs) have been established, as a subpopulation of T cells with regulatory features. Several animal models and patient studies have demonstrated their suppressive functions in many autoimmune diseases. Additionally they are able to inhibit efficient antitumor-immunity. Hence, Tregs display a promising target for therapeutic intervention in several diseases. Nonetheless, the knowledge about the molecular mechanisms underlying their suppressive properties has to be greatly increased, in order to develop novel and innovative Treg-based therapeutic strategies.

Materials and methods: Albeit multiple analyses of the transcriptome of Tregs, little attention has been payed on distinctive signalling pathways cardinally employed by Tregs. Therefore, we ran comparative kinome arrays with Tregs and conventional CD4$^+$ T cells. Our analyses showed that the Casein Kinase 2 (CK2) is one of the most abundantly active kinases in Tregs upon T cell receptor-mediated activation. To further characterize the function of the CK2 in Tregs *in vitro* we used the pharmacological Inhibitor DMAT in classical H3-Thymidin, as well CFSE-Proliferation assays. Additionally we created conditional knockout mice, deficient in the gene of the regulatory CK2-beta subunit, and further analysed the suppressive functions of CK2-beta deficient Tregs in a GvHD model.

Results: Results of our kinome array showed that the Casein Kinase 2 (CK2) is one of the most abundantly active kinases in Tregs upon T cell receptor-mediated activation. Pharmacological inhibition of the CK2, by CK2 inhibitor DMAT, demonstrated a crucial contribution of this enzyme to the suppressive properties of Tregs (unpublished data). In line with this result, conditional genetic ablation of the regulatory CK2-beta subunit underlined the importance of this kinase in Treg-mediated suppression *in vitro* as well as under homeostatic conditions *in vivo*. This finding was further confirmed by a greatly reduced suppressive capacity of CK2-beta deficient Tregs in a GvHD model.

Conclusions: Taken together, our data identify the CK2 as an important kinase essentially involved in the molecular mechanisms underlying Treg-mediated suppression.

P0701

The tumor suppressor gene CYLD controls the function of regulatory T cells

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Purpose/Objective: CYLD is a tumor suppressor gene mutated in familial cylindromatosis, an autosomal-dominant condition that predisposes to multiple skin tumors. In previous reports, CYLD was illustrated to be a negative regulator of the NF-κB pathway by acting as a deubiquitinating enzyme. To analyse the function of CYLD *in vivo* we used the CYLD$^{ex7/8}$ mice, which are characterized by loss of the full-length transcript and overexpression of a short splice variant of *cyld* (sCYLD).

Materials and methods: T cell development and T cell subpopulations in CYLD$^{ex7/8}$ mice were analysed by flow cytometry. The activation of T cells was examined by measurement of proinflammatory cytokines. To adress the suppressive function of CYLD$^{ex7/8}$ Tregs in a physiological context *in vivo*, an adoptive transfer model of colitis was used. The severity of colitis was determined by mini-endoscopy, body weight and histological analysis of the colon. Further, Western blot and RT-PCR were performed to investigate NF-κB signaling in CYLD$^{ex7/8}$ thymocytes and T cells.

Results: The overexpression of sCYLD resulted in splenomegaly and lymphadenopathy. Further, the numbers of mature B cells in spleen and lymph nodes were increased at the expense of T cells. Investigating T cell development in CYLD$^{ex7/8}$ mice we detected a profound impairment in the positive selection of thymocytes, thereby reducing T cells in thymus and periphery. Moreover, CYLD$^{ex7/8}$ T cells were hyperresponsive as a consequence of increased NF-kB activity. Expression of sCYLD markedly increased the numbers of regulatory T cells in thymus and peripheral organs, however the function of these cells was largely diminished accompanied by decreased expression levels of CD25 and CTLA-4.

Conclusions: Our study reveals a crucial role for CYLD in the regulation of T cell development and differentiation as indicated by a significant reduction of T cells in thymus as well as periphery. Interestingly, most of the T cells displayed an effector/memory phenotype. Further, overexpression of sCYLD impaired the *in vivo* immunosuppressive function of Tregs, probably by regulating the expression of CTLA-4 and CD25, which are both involved in the

suppressive function of Tregs. Hence, our data provide evidence for the involvement of sCYLD in controlling Treg cell development and function.

P0703

Transient depletion of regulatory T cells in transgenic mice enhances local immune responses to *Helicobacter pylori*

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Purpose/Objective: Immune suppressive $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Treg) have been shown to accumulate in the stomach during *H. pylori* infection, dampening immunity and contributing to chronicity of infection. The aim of the current study was to address whether *H. pylori* infection in the stomach can be eradicated by boosting immune responses, combining vaccination with transient depletion of Treg.
Materials and methods: Transgenic mice were used in which $Foxp3^+$ Treg co-express the diphtheria toxin receptor (DTR) allowing specific depletion of Treg by injection of diphtheria toxin. Groups of Foxp3-DTR mice were either immunized via the mucosal route with *H. pylori* antigens and cholera toxin adjuvant or left unimmunized. Treg were then transiently depleted at the time of challenge by injection of diphtheria toxin and bacterial colonization, immune responses and inflammation were evaluated 4 weeks post challenge. All comparisons were made between unimmunized and sublingually immunized mice that were either transiently depleted or not depleted of Treg.
Results: In unimmunized *H. pylori* infected mice, depletion of Treg resulted in a significant ($P < 0.05$) decrease in the bacterial load compared to undepleted infection controls. However, regulatory T cell depletion in sublingually immunized mice did not eradicate the *H. pylori* infection; with colonization in the stomach similar to immunized mice not depleted of Treg. Transient depletion of Treg in both sublingually immunized and unimmunized *H. pylori* infected mice was accompanied by significantly enhanced inflammation and CD4 infiltration, and an increase in IFNγ but a reduction in the IL-17 response in the stomach. Finally, depletion of Treg in Foxp3Tg mice without prior *H. pylori* infection resulted in an enhanced CD4 infiltration to the stomach but these cells did not secrete IFNγ or IL-17.
Conclusions: In summary, our results indicate that on of the mechanisms whereby Treg can dampen immunity and thereby promote chronicity of the *H. pylori* infection is by affecting the recruitment of CD4 T cells to the stomach.

P0704

Tribbles-1 is expressed by regulatory T cells and interacts with FOXP3

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Purpose/Objective: We previously identified Tribbles-1 (TRIB1) as a blood and graft biomarker of chronic antibody-mediated rejection in kidney transplant recipients. Although TRIB1 is known to regulate MAPK signaling by interacting with certain MAPKKs, the functional role of TRIB1 in the immune system is still largely unknown. We previously screened different immune compartments and found TRIB1 to be principally expressed by peripheral blood cells including, among others, regulatory T cells (Tregs). We aimed to explore the potential role of TRIB1 in Tregs and to analyze any relationship it may have with Foxp3.
Materials and methods: We isolated $CD4^{pos}CD25^{high}CD127^{low}$ Tregs and $CD4^+$ CD25- T cells by CD4 positive enrichment followed by FACS cells sorting and measured TRIB1 and Foxp3 mRNA by qPCR. Next, physical interaction between TRIB1 and Foxp3 proteins was analyzed in live cells by the Protein Complementation Assay (PCA) in which HEK293 and HeLa cells were co-transfected with plasmids containing the Foxp3 and TRIB1 genes, each comprising a complementary fragment of GFP. Direct physical interactions between the two molecules leading to GFP fluorescence were analyzed by flow cytometry and microscopy. We subsequently analyzed Foxp3 and TRIB1 interaction in the nucleus of freshly-isolated human Tregs. To do this, we isolated nuclear proteins from human Tregs, immunoprecipitated the Foxp3 protein and then performed Western Blotting for the TRIB1 protein.
Results: Both TRIB1 and Foxp3 were expressed at significantly higher levels in Tregs versus their $CD4^+$ CD25- counterparts ($P < 0.001$). Moreover, within Tregs, TRIB1 and Foxp3 mRNA levels correlated tightly (Spearman $r = 1.0$; $P < 0.001$, $n = 7$). The PCA revealed a direct physical interaction between TRIB1 and Foxp3. This interaction took place in the nucleus and had a tendency to decrease upon deletion of the TRIB1 N-terminal (important for nuclear localization) but not the C-terminal. This direct interaction in the nucleus was confirmed in primary human Tregs by the Co-immunoprecipitation analysis.
Conclusions: Overall our results show a relationship between TRIB1 and Foxp3 in terms of their expression and physical interaction, suggesting a role for TRIB1 in Tregs.

Poster Session: Tolerance and Apoptosis

P0705

A unique role of transforming growth factor beta in T cell mediated autoimmunity – modulation of the TCR threshold

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Purpose/Objective: Balancing of the adaptive immunity is an essential prerequisite for maintaining immune homeostasis in vertebrates. During T cell development in the thymus, central tolerance eliminates most self-reactive T cells, which bear TCRs that bind to MHC-self-peptide complexes with high affinity. T cells bearing TCRs with low affinity to MHC-self-peptide complexes regularly escape this process and form the naïve T cell repertoire of the host.However, some of these weakly self-reactive T cells can become activated in the periphery and cause widespread inflammation and destruction of self-tissue culminating in autoimmunity. Thus, peripheral tolerance mechanisms are crucial to maintaining the organism integrity and are not completely understood. Transforming Growth Factor beta (TGFb), is an essential cytokine for regulatory T cells commitment, a subset of CD4 T cells required to maintain peripheral tolerance of T cells. Previous work from our lab suggested that TGFb could also act directly on T cells to maintain tolerance to self. Here, we confirm an essential role for and begin to elucidate the mechanism of TGFb signaling directly on CD8 T cells to maintain tolerance to self.
Materials and methods: Using mice with T cells bearing a defined TCR (F5), either responsive (TGFBRWT), unresponsive (TGFBRKO), or constitutively active TGFb signaling (TGFBRCA) and altered peptide ligands, we clearly established *in vivo* that TGFb signaling controls T cell self reactivity by modulating the TCR activation threshold to MHC-peptides.
Results: In the absence of TGFb signaling, T cells are more reactive to weak affinity peptides, while constitutive TGFb signaling impairs their response to high affinity cognate peptides. In addition, T cells lacking the TGFbR have prolonged contacts with peptide-loaded dendritic cells. Moreover, we observed that TGFb signaling directly controls both the level of phosphorylation down-stream of TCR and the recruitment of Lck to the immunological synapse. Finally, reconstitution of transgenic mice expressing a peptide for the F5 TCR under the MHC-I promoter with bone marrow from F5 TCR Tg TGFBRKO mice leads to massive T cell infiltration.
Conclusions: TGF-b signaling in T cells controls their activation by peptide MHC.

P0706

Cathepsin S dominates autoantigen processing in human thymic dendritic cells

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Purpose/Objective: The interaction of developing thymocytes with peptide-MHC complexes on thymic antigen-presenting cells (APC) is crucial for T cell development, both for positive selection of 'useful' thymocytes as well as negative selection of autoreactive thymocytes to prevent autoimmunity. The peptides presented on MHC II molecules are generated by lysosomal proteases. At the same time, these proteases will also destroy other potential T cell epitopes from self-antigens. This will lead to a lack of presentation on negatively selecting thymic antigen presenting cells and consequently, escape of autoreactive T cells recognizing these epitopes. In order to understand the processes that govern generation or destruction of self-epitopes in thymic APC, we studied the antigen processing machinery and epitope processing in the human thymus.
Materials and methods: The antigen processing machinery of thymic APC was analysed using immunohistochemistry, RT-PCR and active site labeling. *In vitro* digests were performed using lysosomal contents from isolated conventional (mDC) and plasmacytoid dendritic cells (pDC) with myelin basic protein (MBP) and proinsulin as model autoantigens.
Results: We find that each type of thymic APC expresses a different signature of lysosomal proteases, with cortical thymic epithelial cells (cTEC) having a particularly unusual protease pattern. We also find that mDC are more efficient in processing autoantigen than pDC. In addition, we observed that cathepsin S plays a central role in processing of the autoantigens MBP and proinsulin in thymic DC. Cathepsin S destroyed a number of known T cell epitopes, which would be expected to result in lack of presentation and consequently, escape of autoreactive T cells.
Conclusions: The observation that in humans autoantigen processing is dominated by CatS in negatively selecting thymic DC and that CatS destroys known autoantigenic epitopes provides a mechanistic explanation of how autoreactive T cells recognizing these epitopes might escape deletion in the thymus. Cathepsin S therefore appears to be an important factor that influences selection of autoreactive T cells. Furthermore, the unusual and unique MHC II antigen processing machinery that we find in cTEC supports the notion that negative and positive selection occur on different sets of peptides in analogy to what has been described for $CD8^+$ T cells.

P0707

CD28 signaling is also involved in the $CD4^+$ T cell tolerance reaction to harmless exogenous antigen

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Purpose/Objective: The correct decision between an inflammatory immune response versus immune tolerance to a defined antigen is of paramount importance for the survival of the host. CD28 has been described as pivotal for T cell immunity, but its role in a non-inflammatory T cell reaction to 'harmless' antigen, which we recently characterized[1], is unclear. The purpose of this study was to investigate an involvement of CD28 in this tolerance reaction.
Materials and methods: Adoptively transferred OT-II.WT $CD4^+$ T cells were activated by highly purified ovalbumin injected i.v. The role of CD28 was investigated using OT-II.CD28 KO T cells.
Results: Under non-inflammatory conditions, $CD4^+$ T cells de novo upregulate certain surface molecules (e.g. 4-1BB, CD160, RANKL), which are not expressed by T cells under inflammatory conditions. In addition, these 'non-inflammatory response T cells' (T_{nir}) produce IL-2 and other non-inflammatory cytokines, and proliferate. This early reaction to pure antigen leads to a threefold initial expansion of the T_{nir} population on day 2.5. Gene expression analyses and studies at the protein level revealed that CD28 has a dual role in the T_{nir} setting. On the one hand, absence of CD28 prevented upregulation of IL-2 and abrogated the expansion of T_{nir} cells, indicating that CD28 signaling has an essential role in the normal T_{nir} reaction to harmless environmental antigens, which finally leads to tolerance[1]. On the

other hand, absence of CD28 resulted in further upregulation of T_{nir}-specific cell activation molecules like 4-1BB and CD160, indicating a role of CD28 in the suppression of some T_{nir} reaction elements under non-inflammatory conditions.

Conclusions: Taken together, these data reveal a complex role of CD28 *in vivo*. CD28 thus not only acts as a potent T cell costimulator under inflammatory conditions, but also functions when harmless environmental antigens are presented to T cells. This conclusion extends the current paradigm, in which CD28 is exclusively regarded as a central mediator of the inflammatory T cell response.

1. Lischke, Hegemann et al. Comprehensive analysis of $CD4^+$T cells in the decision between tolerance and immunity *in vivo* reveals a pivotal role for ICOS. 2012. *J Immunol*, in press.

P0708

Description of the HLA class I peptide repetoire in the human thymus

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Purpose/Objective: The thymus is the organ where T lymphocytes mature and differenciate. For that, thymocytes must contact with antigen presenting cells through the interaction between the TCR and self peptides complexed with self HLA molecules. The peptide repertoire associated to HLA molecules in the thymus must mirror the repertoire that T cells will see in periphery, including the presence of peptides derived from tissue restricted antigens. So far the composition of the human peptidoma associated to HLA class I molecules remains unknown. The objective of the present work was to describe the HLA class I peptide repertoire from human thymus.

Materials and methods: Human thymus samples from children who underwent corrective heart surgery were lysed, peptide-HLA-I complexes were purified by immunoaffinity chromatography and the peptide pool was purified by ultrafiltration. Peptide sequences were obtained by LC-MS.

Results: A total of 230 HLA class I natural ligands were identified. The peptide pool was composed by high affinity HLA class I ligands. These peptides derived from proteins principally located in the cytosol and nucleus. Nineteen peptides were found in different thymus samples and some proteins generated more than one peptide. Finally, a peptide of the protein SPATIAL, with restricted tissue expression was identified.

Conclusions: An extensive analysis of the peptidoma bound to HLA class I molecules is presented here. This peptide pool has similar features to the peptide repertoires of other tissues: high affinity ligands derived from cytosolic and nuclear proteins. The presence of some peptides in several samples suggests an immunodominance during the processes of central tolerance. Finally, a peptide with restricted expression was identified, what can be relevant in the generation of central tolerance against the protein SPATIAL.

P0709

Effect of HCV-core expression in $CD4^+$ primary Lymphocytes

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Purpose/Objective: Hepatitis C virus (HCV) infection is a worldwide health problem that affects more than 170 million patients and evolves into chronicity in over 85% of cases.

It has been shown that failure of an effective cellular response is pivotal to explaining viral persistence and accumulating evidence suggests that HCV (Hepatitis C virus) proteins suppress host immune responses by interfering in the function of immune cells, with HCV core being one of the most active.

It has previously been shown by us and others that HCV-core induces suppression when expressed in the $CD4^+$ tumor T cell line Jurkat the NK cell line YTS, or when added to $CD4^+$ T cell cultures.

As there is evidence indicating that HCV can be present in cells either than the hepatocyte, including $CD4^+$ lymphocytes, we intend to analyze the effect of HCV-core expression in $CD4^+$ primary lymphocytes.

Materials and methods: $CD4^+$ primary lymphocytes immunomagnetically purified from peripheral blood mononuclear cells (PBMC) were transduced with lentiviral supernatants containing an HCV-core-GFP expression cassette.

Results: HCV-core transduced $CD4^+$ T cells acquire a regulatory phenotype upregulating Foxp3 and CTLA4 and suppressing the response of bystander T cells.

Conclusions: Our results add a new element in this cell population, and give an example of the generation of $CD4^+$ T cells with regulatory activity by the expression of a single viral protein.

P0710

Exploring the basis of T cell selection: natural MHC class I and II peptides presented by dendritic cells in the human thymus

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Purpose/Objective: Central tolerance to self is imposed on T cells during their development in the thymus by interaction of the T cell receptor with peptide-MHC complexes displayed by thymic dendritic cells (DC) or medullary thymic epithelial cells (mTEC). T cells recognizing these ligands with high affinity will be deleted before they can escape into the periphery and cause autoimmune disease, a process called negative selection. However, the nature of the self-peptides on which negative selection occurs in the human thymus is entirely unknown.

Materials and methods: MHC class I and II molecule-peptide complexes were isolated by affinity chromatography from whole thymus, isolated DC and DC-depleted samples, bound peptides eluted with acid and subsequently analysed by mass spectrometry.

Results: We identified several hundred peptides in our samples, including a large number of ligands presented by negatively selecting dendritic cells. Analysis of eluted peptides revealed a significant degree of cross talk between MHC I and II pathways and the presence of both autoimmune and cancer-related epitopes.

Conclusions: We here provide the first data on the MHC-peptide matrix on which T cell selection occurs in the human thymus.

P0711

Flow cytometry-based method for detection of caspase dependent degradation of linker for activation of T cells

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Purpose/Objective: Caspase/Granzyme B mediated protein degradation is involved in elimination of activated T cell receptor (TCR) signaling molecules during processes of thymocyte selection and maintenance of peripheral homeostasis of T cells. Key components of TCR signaling cassette e.g. TCR ζ chain, phospholipase C-γ1, proto-oncogene product Vav1, hematopoietic progenitor kinase 1, adaptor protein GADS undergo biological inactivation in response to pro-apoptotic or anergy inducing environmental stimuli. Although available Western immunoblotting-based techniques are appropriate for detection of protein degradation in bulk populations of target cells, quantitative assessment of this process at single cell level requires a different approach.
Materials and methods: We report on a novel, flow cytometry-based method for assessment of LAT integrity.This method exploits a loss of an anti-LAT antibody epitope recognition following caspase dependent proteolytic degradation of C-terminal domain of the LAT adaptor.
Results: We showed that upon physiological inputs including TCR engagement, Fas ligation, glucocorticoid hormone receptors stimulation or treatment with chemical apoptosis inducers, such as etoposide, staurosporine or ionomycin, COOH-terminal domain of LAT undergoes rapid degradation leading to a loss of an anti-LAT antibody epitope. The kinetics of this degradation process correlates with reported rates of caspase activation and in its initial phase precedes the phosphatidylserine translocation to the outer leaflet of the plasma membrane. As a result of the degradation, a NH2-terminal membrane spanning fragments of LAT are detectable. A most prominent of them the 17kDa fragment matches a peptide generated by *in silico* cleavage of a caspase target sequence present at position 160–164 (DLGD) of the mouse LAT. The screening of protease inhibitors indicated that caspase 8 is in part responsible for the degradation of LAT but other proteases, in particular serine protease(s) whose activity is blocked by PMSF may also be involved.
Conclusions: In contrast to available Western immunoblotting based approaches requiring millions of purified cells to meet sensitivity criteria, our method allows for single cell level assessment of LAT degradation in discrete populations of cells defined by multi-parameter flow cytometry. By assessing the integrity of LAT our approach offers insight into physiological processes attenuating TCR signaling by selective decomposition of the LAT signalosome.

P0712

In vitro-differentiated pDCs suppress costimulation blockade mediated DTH response in absence of CCR7-signalling

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Purpose/Objective: CCR7 is an important molecule for homing of naïve T cells to lymph node (LN). CCR7 is also necessary for homing of Treg cells into peripheral LNs and that this homing is a prerequisite for their *in vivo* function. Plasmacytoid dendritic cells (pDCs) are suggested to behave as antigen presenting cells in settings of tolerance induction such as oral tolerance and allograft survival. CCR7$^{-/-}$ mice harbour significantly reduced numbers of pDC within LNs, but not bone marrow or spleen. According to our recent findings, CCR7$^{-/-}$ recipients fail to prolong survival of cardiac allografts in a model of allotolerance induction by donor splenocyte transfusion (DST) in combination with anti-CD40L-mAb mediated costimulation blockade (CSB). Strikingly, adoptive transfer of syngeneic wild type pDCs was indeed sufficient to rescue graft survival in DST^{+}CSB-treated CCR7$^{-/-}$ recipients.
Materials and methods: Recently, we were able to standardize *in vitro* pDC differentiation protocol, by using Flt-3L directly sequestered into cell culture supernatant by B16FL melanoma cells. Preliminary results indicate that these *in vitro* pDCs are highly functional in inducing Tregs *in vitro*. In order to investigate the tolerogenic potential of these *in vitro* differentiated pDCs we standardised a delayed type hypersensitivity (DTH) model in CCR7$^{-/-}$ recipients.
Results: It is already known that LNs of CCR7$^{-/-}$ mice are devoid of T cells and DTH response to a T cell dependent antigen is delayed in these mice. However, we could observe DTH response when CCR7$^{-/-}$ mice were immunized with our earlier described allotolerance induction protocol of DST^{+}CSB. The recipients were subsequently challenged with allogenic splenocytes lysate emulsified together with complete freund's adjuvant (CFA). We further sorted pDC fraction from the conventional dendritic cell (cDC) fraction and adoptively transferred together with DST^{+}CSB to analyse the DTH response. We could observe that syngenic *in vitro* differentiated immature pDC but not cDC could suppress the allogen mediated DTH in CCR7$^{-/-}$ mice. On the other hand, TLR7/8 agonist, R848-stimulated pDCs fail to suppress the DTH response in CCR7$^{-/-}$ mice.
Conclusions: These observations further validates that pDC could act as immunogenic cell sentinels and also as tolerogenic cells which mediate Treg development and/or suppression of self- and allo-reactive cells.

P0713

Increased antibody levels against gut commensals are associated with regulatory T cell defect in patients with APECED

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Purpose/Objective: APECED is a rare monogenic autoimmune disease, which is caused by a loss-of-function mutation in the Autoimmune Regulator (AIRE) gene. APECED is characterized by chronic mucocutaneous candidiasis and autoimmunity to multiple endocrine organs. Taken into consideration the gastrointestinal symptoms of the disease and the emerging role of commensals in controlling the immune system we set out to study the handling of commensal microbes and their effects on the immune system in patients with APECED.
Materials and methods: Plasma antibody levels against several commensal microbes were measured in 12 APECED patients and 26 healthy controls using an ELISA assay. The antibody levels were compared to T-cell data acquired using flow cytometry.
Results: The patients showed significantly elevated IgG responses against *Saccharomyces cerevisiae*, *E. coli* and *Pseudomonas fluorescens* displaying a Crohn's disease-like loss of tolerance to commensals. Remarkably, the antibody levels against *S. cerevisiae* showed considerable negative correlation to naïve recent-thymic emigrant regulatory-T-cell population and FOXP3 expression of Tregs creating a link between the commensal responses and impaired regulatory T cell function seen in APECED.
Conclusions: Our data on the elevated antibody levels against commensals are the first to depict loss of tolerance against non-self antigens in APECED. Moreover, the correlation of anti-commensal antibody levels to diminished Treg function creates a completely new approach to APECED disease pathogenesis and to the crosstalk of commensals and immune system.

P0714

Induction of allograft tolerance by monoclonal CD3 antibodies: a matter of timing

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Purpose/Objective: Despite remarkable progress in organ transplantation through the development of a wealth of immunosuppressive drugs highly effective at controlling acute ejection, two major problems still remain, the loss of grafts due to chronic rejection and the growing number of sensitized recipients due to previous transplants, transfusions or pregnancies. Induction of immune tolerance appears to be the only way to curb this complex situation. We have previously shown in autoimmunity that CD3 antibody therapy promotes tolerance only when applied in the context of a primed immune system. We sought to extend this observation to the transplant setting.

Materials and methods: Pancreatic islets from BALB/c mice were grafted under the kidney capsule of streptozotocin-treated diabetic C57BL/6 recipients. Five injections of Fc non-binding CD3 antibody $F(ab')_2$ fragments (50 μg/injection/day) were administered at the time (day-1) or after transplantation (day$^+$7 or 11). Graft survival was monitored by blood glucose measurements.

Results: We demonstrate that a short-term, low-dose course with CD3 antibodies starting on day$^+$7 or$^+$11 after transplantation induces long-term survival (>100 days) of fully mismatched islet allografts. Importantly, permanent acceptance of second islet grafts from the original but not third party donors proved that antigen-specific tolerance had indeed been induced in these recipients. Mechanistic studies revealed that antigen-specific regulatory and effector T cells are differentially affected by the treatment. CD3 antibody treatment preferentially induces apoptosis of activated alloreactive T cells which is mandatory for tolerance induction. As a consequence, anti-donor CD8 responses are dramatically reduced in tolerant hosts. In contrast, regulatory T cells are relatively spared from CD3 antibody-induced depletion and can transfer antigen-specific tolerance thus arguing for their prominent role in sustaining long-term graft survival.

Conclusions: Our data show that judicious use of CD3 antibodies can induce transplant tolerance to fully mismatched allografts in recipients primed to the alloantigens. From the translational point of view and due to the availability of humanized CD3 antibodies presently developed in autoimmmune diabetes, our finding provides further consideration of using CD3 antibody-based therapy in transplantation

P0715

Induction of anergic T cells by CD83highCCR7highHLA-DRhigh as well as CD83lowCCR7negativeHLA-DRlow IL-10-modulated human dendritic cell subpopulations

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Purpose/Objective: Our studies previously demonstrated that IL-10-modulated tolerogenic human dendritic cells (IL-10DC) induce anergic CD4$^+$ T cells. Here, we investigated both, CD83^{high}CCR7^{high}HLA-DR^{high} and CD83^{low}CCR7^{negative}HLA-DR^{low} IL-10DC subpopulations, with regard to their phenotype and tolerogenic capacity to generate anergic CD4$^+$ T cells in detail.

Materials and methods: Therefore, we compared the expression of costimulatory and inhibitory molecules of the B7- and ILT-family, respectively, between human mature DC (mDC) and both IL-10DC subpopulations. FACS sorting of both IL-10DC subpopulations was performed with regard to the high or absent expression of CCR7 (representing CD83^{high} or CD83^{low} DC). Subsequently, coculture experiments with naïve CD4$^+$ CD45RA$^+$CD25^{low} T cells were conducted.

Results: As compared to mDC, the CD83^{low} IL-10DC subset exhibited a significantly reduced expression of costimulatory molecules (CD80, CD86, B7-H2, CD40) accompanied by a slight upregulation of inhibitory molecules (B7-H1, ILT3, ILT4). In contrast, we observed minor changes in expression of costimulatory molecules but significantly enhanced levels of inhibitory molecules on CD83^{high} IL-10DC, demonstrating significant differences in expression of costimulatory and inhibitory molecules (B7-H1, B7-DC, ILT3) between the two IL-10DC subsets.

Notably, primary stimulation and restimulation experiments revealed that both subpopulations, in contrast to mDC and regardless of their maturation state, induced anergic CD4$^+$ T cells as evidenced by a significantly reduced T cell proliferation and diminished Th1 and Th2 responses (T-bet, IFN-γ; GATA-3, IL-5, IL-13).

Conclusions: In conclusion, both phenotypic mature CD83^{high} CCR7^{high}HLA-DR^{high} and immature CD83^{low}CCR7^{negative}HLA-DR^{low} IL-10DC subpopulation display properties of tolerogenic human DC which may be used for the development of novel therapeutic approaches for allergies, autoimmune disease or transplant rejections.

P0716

Involvement of human suppressor and non-suppressor FOXP3+ T cells in HIV-1 infection

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Purpose/Objective: FOXP3 is a key transcription factor for the development and function of regulatory T cells (Tregs); however, it can also be expressed by non-suppressor T cells. In the present study, we examined the different behaviors of FOXP3+ Tregs and FOXP3+ non-Tregs in HIV-1 infected patients.

Materials and Methods: PBMCs isolated from HIV-1 infected (n = 55) and healthy donors (HD, n = 27) were analyzed by flow cytometry to characterize five subsets of CD4+ T cells: CD45RA+ FOXP3^{low} (rTregs), CD45RA- FOXP3^{high} (aTregs), CD45RA- FOXP3^{low} (FOXP3+ non-Tregs), CD45RA+ FOXP3- (naïve) and CD45RA- FOXP3- (memory). Frequency and absolute number of each subset were compared and correlated with CD4 count, viral load (VL) and activation markers (HLA-DR and CD38). Moreover, *in vitro* susceptibility of different HD purified subset cells to HIV-1 BaL was assessed by flow cytometry and ELISA. Cytokine levels were evaluated using a multiplex approach.

Results: In addition to the absolute decrease of conventional T cells, patients present a significant decrease in all FOXP3+ T cells. aTregs from HIV-1 patients did not correlate with the CD4 count and none FOXP3+ T cells correlated with VL. Furthermore, an increase immune activation that inversely correlated with FOXP3+ non-Tregs was observed in those patients with lower CD4. We confirmed the HIV-1 infection of all CD4+ cells, presenting higher levels in aTregs, FOXP3+ non-Tregs and memory T cells. FOXP3+ non-Tregs have secreted a wide spectrum of Th1, Th2 and Th17-like cytokines in HD. Moreover, we found a dramatic decrease of Th1 and Th17-like cytokine secretion post infection of FOXP3+ non-Treg, polarizing towards a Th2 cytokine secretion.

Conclusions: In chronically infected HIV-1 patients, we found a decreased absolute number of suppressor aTregs and FOXP3+ non-Tregs. It still remains to be elucidated if the decreased number of suppressor Tregs plays a role as suppressor of the anti-viral immune

response or impacts on the immunological activation phase favouring virus replication. On the other side, our results establish a new basis for the reliable characterization of the role of FOXP3+ T cells in HIV-1 individuals.

P0717

Phagocytosis of infected apoptotic cells promote PGE2 production by dendritc Cells

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Purpose/Objective: Phagocytosis of apoptotic cells promotes the synthesis of anti-inflammatory mediators such as PGE_2, TGF-β and IL-10 that may result in the suppression of host immune defense. However, a study using infected apoptotic cells showed that phagocytosis of these by dendritic cells (DC) promotes the production of anti-inflammatory cytokines such as TGF-b but also pro-inflammatory cytokines as IL-6 and IL-23, resulting in an immunostimulatory effect, the differentiation of Th17 cells. The role of PGE_2 in adaptive immunity has been investigated regarding lymphocyte differentiation and activation. Our aim was to evaluate the PGE_2 production from DC when co-cultured with different ratios of infected apoptotic cells.
Materials and methods: Infected apoptotic neutrophils were prepared as follows: C57BL/6J mice were injected i.p. with 3 ml thioglicollate with 3×10^6 live *Escherichia coli*, after 13 h, the cells were collected from peritoneal cavity lavage. Apoptosis of neutrophils were confirmed by flw cytometry using Annexin-V/PI staining. Bone marrow derived dendritic cells were co-cultured at different ratios (1:1, 1:3, 1:5) with infected apoptotic neutrophils during 18 h. Supernatant from co-culture was collected and PGE_2 production was determined by ELISA. We also evaluated the maturation level of DC after co-culture.
Results: Our results show that phagocytosis of infected apoptotic cells induces the production of high levels of PGE_2 at 1:3 (1500 pg/ml) and 1:5 (3400 pg/ml) ratio. Interestingly, the DC co-cultured with infected apoptotic cells deviated for immature state, with low levels of CD11c, MHC-II and CD86, contrasting with the activating stimulus LPS. Furthermore, the proportion of phenotypically immature cells increased in higher infected apoptotic cells rates.
Conclusions: DC does not acquire a predominant activated state when co-cultured with infected apoptotic cells and further characterization are needed to understanding this phenotypical state and its role in Th17 development. Also, the higher levels of PGE_2 suggest a role for this lipid mediator in Th17 differentiation in this context. However, the involvement of PGE_2 and the mechanism by which PGE_2 can work synergistically with TGF-b, IL-6 and IL-23 in the process of Th17 cell differentiation needs further characterization.

P0718

Phenotypic and functional characteristics of splenocytes in tetanus toxoid-hyperimmunized Balb/c mice is influenced by the context of tetanus toxoid application

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Purpose/Objective: The hyperimmunization with tetanus toxoid (TTd) induces protective TTd-specific as well as autoreactive β2-glycoprotein I (β2GPI)-specific immune responses in BALB/c mice. The overall immune response characteristics, especially its pathogenic potential, depended on adjuvants applied prior and in combination with TTd. Beside structural homology between TTd and β2GPI, tolerance toward β2GPI could be impaired by adjuvants acting as polyclonal stimulators. In order to clarify the impact of adjuvants, phenotypic and functional analyses of immune system cells within spleen were done upon immunization completion.
Materials and methods: Non- or CFA-pretreated BALB/c mice were immunized with TTd (3×100 μg/dose; 2-week intervals) mixed with alum or 2.5M glycerol. *Ex vivo* analyses of CD3, CD4, CD8, CD19, CD 25, CD27 and mIgM expression on age-matched control and immunized mice's splenocytes were done by flow cytometry. Changes in TLR2, TLR4 and TLR9 expression were assessed indirectly, by measuring cytokine production, following *in vitro* stimulation of splenocytes with appropriate agonist.
Results: TTd-immunization diminished CD27 expression on T cells implying on their differentiation into potent effector cells. T cell activation (increase in CD25 expression and the raise of percentage of $CD4^+$ $CD8^+$ $CD3^+$) and B cell activation (rise in percentage of $CD19^+$ $CD25^+$ cells and the increase of mIgM density) occurred in all immunized mice, being more intensive in CFA-pretreated groups. Irrespective to the applied immunization protocol, statistically significant rise in abundance of CD4- CD8- cells (often cited as cells having suppressive potential) within T cell pool was registered too. Differences in cytokines production (IL4, IL10, IFNγ) registered upon *in vitro* stimulation with peptidoglycan, LPS and CpG ODN implied on context-dependant modulation of TLR2, TLR4 and TLR9 expression on splenocytes.
Conclusions: TTd-hyperimmunization promoted concomitant rise in abundance of activated cells and the cells that have suppressive potential. This could be regarded as an attempt of the system to retain control. Imbalance in percentages and activities between activated cells and those having suppressive potential, highly influenced by the context of TTd application, is most likely the cause for the observed pathology appearance after TTd hyperimmunization.

P0719

Processing of particle bound antigens by peripheral phagocytes induces immunological tolerance

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Purpose/Objective: Phagocytes are key players in the upkeep of body homeostasis, e.g. by removing apoptotic material as well as forming a primary line of defense against invading microbiota. In this study we investigated the effect of systemically distributed particle bound antigens under sterile conditions to address the immunoregulatory capacities of phagocytes throughout the body in homeostasis.
Materials and methods: 0.5 μm fluorescent latex particles were covalently linked to ovalbumin (OVA) and injected i.v. into recipient mice. Latex particle distribution was followed up to 14 days in wildtype, CX3CR1.eGFP and CCR2.eGFP mice using flow cytometry and multiphoton microscopy to investigate uptake by distinct phagocyte subsets. Bone marrow transplants using CFP^+ donors were performed to assess latex particle distribution between resident macrophages and monocyte-derived phagocytes. Tolerance induction was measured by activation of regulatory T cells (Treg) and inhibition of OVA-specific CTL mediated target cell lysis. Interactions between latex-positive phagocytes and T cells were followed using intravital time lapse multiphoton microscopy in liver tissue as well as secondary lymphatic organs.

Results: Injection of OVA-latex particles (OVA-Lx) lead to a significant reduction in OVA-specific CTL lysis. Furthermore, OVA-Lx treatment induced expansion and enhanced activation of regulatory T cells. Latex particles were most efficiently taken up by liver resident phagocytes (resembling Kupffer cells) but not monocyte derived macrophages and were almost undetectable in secondary lymphatic tissues and bone marrow. Latex beads could be detected for up to 14 days after injection and did not redistribute significantly during that time period.
Conclusions: Peripheral phagocytes such as liver Kupffer cells provide not only a mechanism for clearing small sized particles from the circulation but can also act as efficient inducers of regulatory T cell activation, therefore providing a potential key mechanism in the upkeep of self-tolerance under homeostatic conditions.

P0720
Regulation of thymocyte negative selection by IL-15Ra and IL-15

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Purpose/Objective: Central tolerance mechanisms operated during T cell development in the thymus handle the self-reactive T cell repertoire generated by TCR gene rearrangement. Thymocytes with TCR exceeding certain level of affinity for self-antigens either die by negative selection or differentiate into regulatory T (Treg) cells through interacting with self-antigens presented by medullary thymic epithelial cells and dendritic cells. Interleukin-15 and its receptors were reported to regulate the development of thymic NKT cells. However, the role of IL-15 system in the development of the majority of thymocytes remained unclear.
Materials and methods: By using TCR transgenic mice, we developed several experimental models to investigate the function of thymus negative selection in WT or IL-15 system deficient mice.
Results: Here we show that the deficiency of IL-15Ra and IL-15 in these antigen-presenting cells (APC) impaired thymocyte negative selection by self-antigen in the relatively low negative selection affinity range. While the development and function of naturally occurring Treg cells are normal in *Il15ra*$^{-/-}$ and *Il15*$^{-/-}$ mice. The mutant mice also developed autoimmune symptoms.
Conclusions: These results indicate that the IL-15 system of thymic APCs is required for optimal negative selection of thymocytes and thus regulation of T cell-mediated autoimmunity.

P0721
Role of the chemokine CCL2 in T cell tolerance

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Purpose/Objective: CCL2 is an inflammation-associated chemokine inducing the recruitment of immune cells to tissues. CCL2 and its receptor CCR2 are implicated in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Mice lacking CCL2 or CCR2 are resistant to EAE.

We observed that transgenic mice expressing CCL2 in central nervous system (MBP/CCL2) were resistant to EAE. MBP/CCL2 crossed with 2D2 mice, whose T cells express the transgenic Vα3.2β11 T cell receptor (TCR) specific for the self-antigen myelin oligodendrocyte glycoprotein (MOG), were also resistant to EAE. Both MBP/CCL2 and 2D2 × CCL2 mice also showed ectopic expression of CCL2 in thymus. Our goal was to investigate whether thymic expression of CCL2 tolerized encephalitogenic T cells.
Materials and methods: Thymus and lymph node (LN) from C57BL/6 and transgenic mice were analyzed by flow cytometry LN from MOGp35-55 immunized mice were cultured with 10 μg/ml MOG peptide. After 4 days, cell proliferation was measured by CFSE dilution Stromal cells and thymocytes were separated using a Percoll gradient (densities 1.065 and 1.115) from thymi treated with TrypLE and DNase Transcripts were measured by quantitative PCR.
Results: We observed that in mice expressing CCL2 in thymus, LN cells proliferated poorly in response to MOG peptide *in vitro*. In 2D2 × CCL2 mice, transgenic Vα3.2$^+$ CD4$^+$ T cell development was severely impaired. Vα3.2$^+$ CD4$^+$ T cells were lost in both thymus and LN. We also showed that CCL2 transcripts were expressed in thymic stromal cells, which are important for T cell development and auto-reactive T cell deletion. We thus hypothesized that CCL2 was involved in regulation of auto-reactive T cell development. When we crossed MBP/CCL2 with OT2 mice, whose T cells express the transgenic Vα2β5 TCR specific for the non-self-antigen ovalbumin, CCL2 transcripts were not expressed in thymus and Vα2$^+$ CD4$^+$ T cells were relatively unaffected.
Conclusions: We conclude that in CCL2 transgenic mice, interaction between auto-reactive thymocytes and thymic stromal cells induces ectopic expression of CCL2, and that CCL2 expression in thymus is involved in impairment of auto-reactive T cell development which leads to protection against EAE. This identifies a novel role for CCL2.

Our goal now is to investigate thymic stromal cells in order to determine how auto-reactive T cells are impaired.

P0722
Stroma cells in the modulation of immunity and tolerance in the intestinal immune system

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Purpose/Objective: Mucosal tolerance is best studied in the intestinal immune system. Importantly, the effects of oral tolerance are not limited to intestine but act body-wide, actively inhibiting cellular and humoral immune responses, indicating that oral tolerance might be exploited therapeutically e.g. to treat autoimmune diseases. Low doses of antigen have been suggested to act primarily by inducing regulatory T cells, whereas high doses of antigen have been suggested to cause cell depletion by apoptosis and/or anergy of antigen-specific T cells.

Fibroblastic reticular cells (FRCs) are one of the main populations of nonhematopoietic stromal cells in lymph nodes. FRCs are also known to maintain the homeostasis of naïve T cells. Therefore, any effect of FRCs on activated T cells is highly significant.

Thus, the objective of this study is to investigate the role of stromal cells in T cell homeostasis.
Materials and methods: Either MLN or PLN fragments are grafted into the gut mesenteries after excision of endogenous MLN. Eight weeks after transplantation OT II Ly5.1 cells are adoptively transferred followed by oral Ova administration and intracellular staining to study expression of transcription factors for various CD4$^+$ T cell subsets is done on D5 after transfer.
Results: We observed higher secretion of FoxP3 in TxMLN as compared to TxPLN. Also our preliminary results suggested higher expression of T-bet in TxMLN in comparison to TxPLN while opposite effect was observed for Gata-3.
Conclusions: Stromal cells determine tolerogenic properties of transplanted LN.

P0724

Survival and immunosuppressive functions of myeloid-derived suppressor cells are not regulated by the CD95/CD95L system

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Purpose/Objective: Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of immature CD11b-expressing precursor cells, which accumulate in pathologic conditions. Depending on the expression of Ly-6C or Ly-6G, they are divided into two subpopulations: monocytic MDSCs (Ly-6C^{high}Ly-6G^{neg}) and granulocytic MDSCs (Ly-6G^{high}Ly-6C^{low}). Both subpopulations suppress T cell activation and proliferation. During this process they are in direct contact with CD95L-expressing T cells, able to induce apoptosis in CD95 expressing cells. Therefore, we analyzed apoptosis sensitivity of MDSCs after T cell contact in order to clarify whether apoptosis resistance might be a possible mechanism by which MDSCs survive T cell attacks and induce immune suppression.
Materials and methods: MDSCs were generated by culturing bone marrow cells for 4 days in the presence of GM-CSF. Both subpopulations were isolated by magnetic beads and expression of death receptor CD95 was defined by flow cytometry. CD95-sensitivity and apoptosis induction of MDSCs was analyzed by treating cells with recombinant CD95L, by co-culturing MDSCs with isolated T cells or by adding MDSCs in mixed lymphocyte reactions (MLR). Further, we analyzed whether usage of MDSCs derived from CD95 deficient lpr mice can increase the suppressive capacity of MDSCs.
Results: Granulocytic and monocytic MDSC subpopulations exhibited a comparable expression of CD95 and both underwent apoptosis in the presence of recombinant CD95L. Apoptosis induction was associated with activation of caspase-8 and -3 and was blocked by the caspase inhibitor zVAD-fmk. However, after co-incubation of MDSCs with CD95L-expressing naïve T cells no apoptosis induction of MDSCs was detected. Also, when MDSCs were added to allogeneic MLRs, they efficiently inhibited proliferation of T cells in a dose-dependent manner and did not undergo apoptosis. Usage of MDSCs from CD95 deficient lpr mice in allogeneic MLRs did not increase the inhibition of allogeneic T cell proliferation compared to CD95-expressing MDSCs. CD3/CD28- activated T cells could induce cell death in MDSC, however, in a CD95L-independent way.
Conclusions: Although MDSCs express CD95, they are insensitive to CD95L-mediated T cell-induced apoptosis showing that homeostasis and suppressive capacity of MDSC is not regulated by the CD95/CD95L system.

P0725

T cell apoptosis and induction of Foxp3$^+$ regulatory T cells underlie the therapeutic efficacy of CD4-blockade in experimental autoimmune encephalomyelitis

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Purpose/Objective: The pathogenesis of multiple sclerosis (MS) requires the participation of effector neuroantigen-specific T cells. Thus, T cell targeting has been proposed as a promising therapeutic strategy. However, the mechanism underlying effective disease prevention following T cell targeting remains incompletely known.
Materials and methods: We used different TCR transgenic mouse strains, namely MBP-specific and MOG-specific, to evaluate the mechanism underlying the effect of non-depleting anti-CD4 monoclonal antibodies (MAb) in protecting the mice from spontaneous and induced experimental autoimmune encephalopathy (EAE).
Results: We found, using several TCR-transgenic strains, that CD4-blockade is effective in preventing EAE and in treating mice after the disease onset. The mechanism does not rely on direct T cell depletion, but the anti-CD4 MAb prevents the proliferation of naïve neuroantigen-specific T cells, and acquisition of effector Th1 and Th17 phenotype. Simultaneously, the MAb favors peripheral conversion of Foxp3$^+$ Treg cells. Pre-existing effector cells, or neuroantigen-specific cells that undergo cell division in spite of the presence of anti-CD4, are committed to apoptosis.
Conclusions: Therefore, protection from EAE relies on a combination of dominant mechanisms grounded on Treg cell induction, and recessive mechanisms based on apoptosis of neuropathogenic cells. We anticipate that the same mechanisms may be implicated in other T cell-mediated autoimmune diseases that can be treated or prevented with antibodies targeting T cell molecules, such as CD4 or CD3.

P0726

The pre-B cell receptor; censoring the development of autoreactive B cells

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Purpose/Objective: The surrogate light chain (SLC) is assembled from the VpreB and l5 polypeptides, encoded by genes that are expressed specifically in pro- and pre-B cells. During B cell development, functional recombination of the Ig heavy chain (HC) locus, in pro-B cells, leads to the assembly of mHCs with the invariant SLC, which results in the formation of a pre-B cell receptor (pre-BCR), transition to the pre-B cell stage and expansion by proliferation. We have previously found that cells that can potentially develop into autoreactive B cells are negatively selected at this transition: in mice that lack SLC (SLC$^{-/-}$), autoreactive B cells develop as well as plasma cells secreting autoantibodies typical of autoimmune diseases, e.g. systemic lupus erythematosus (SLE). Here, we have investigated the SLC$^{-/-}$ mice in greater detail with focus on the autoreactive B cells and the autoantibodies.
Materials and methods: SLC$^{-/-}$ and control mice were analysed to determine e.g. serum autoantibody levels, antigen specificity, antibody sequences, and cellular composition of lymphoid tissues by flow cytometry and immunohistochemistry, etc.
Results: Unexpectedly, we find that the B cell population with autoreactive characteristics present with a phenotype reminiscent of germinal centre B cells. In support of this germinal centres, typical of T cell-dependent immune responses, are detected by immunohistochemistry in 4–5 month old SLC$^{-/-}$ but not control mice. Around this time we detect plasma blasts/cells by flow cytometry SLC$^{-/-}$ and find that the levels of IgG autoantibodies are significantly elevated whereas those of IgM are increased already at 2–3 month. These observations are being further investigated.
Conclusions: Collectively, our findings demonstrate that lack of negative selection at the pre-BCR checkpoint allow for spontaneous germinal centre reactions and the production of IgG autoantibodies. These data argue that tolerance checkpoints later in B cell development are not sufficient or, alternatively, cannot fully replace that of the pre-BCR. They also imply that a B cell-intrinsic defect at the pro- to pre-B cell transition may have a detrimental effect on control of peripheral autoimmune reactions.

P0727

The regulation of immediate early response gene IEX-1 expression

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Purpose/Objective: Identifcation of mechanisms of regulation of *IEX-1* expression by EGF and identification of proteins engaged in IEX-1 transcript turnover. *IEX-1* is an immediate early gene which expression is induced rapidly and transiently upon stimulation with growth factors, inflammatory cytokines, LPS or PMA. The protein product of the gene is involved in the regulation of apoptosis and can positively as well as negatively affect the cell survival. IEX-1 is also engaged in oncogenesis.
Materials and methods: human hepatoma HepG2 cell line, reporter vectors containing *IEX-1* promoter variants or IEX-1 3′UTR, luciferase essays, Northen blots, Western blots.
Results: Expression of *IEX-1* is regulated by both EGF and PMA in ERK1/2-dependent manner. EGF stimulation accompanied by ERK1/2-dependent phosphorylation of transcription factor Elk-1 suggests that Elk-1 may activate *IEX-1* transcription. Using reporter system with luciferase gene under the control of the *IEX-1* promoter we have shown that Elk-1 activates *IEX-1* promoter and that this regulation is SRF-independent. By series of genetic constructs containing variants of *IEX-1* promoter we have found that the region between -140 and -95 bp is essential for *IEX-1* promoter activity.

Moreover, using the reporter gene construct containing luciferase gene with attached *IEX-1* 3'UTR we have identified proteins possibly engaged in the regulation of IEX-1 mRNA half-life. These proteins include newly identified RNase MCPIP-1 which shorten the IEX-1 mRNA half-life and HuR involved in stabilization of this transcripts.
Conclusions: EGF regulates *IEX-1* expression through the activation of transcription factor Elk-1. The half-life of IEX-1 mRNA is regulated by MCPIP-1, TTP and HuR.

P0728

The role of calpain-calpastatin system in reduced apoptosis of childhood acute lymphoblastic leukemia cells

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Purpose/Objective: Acute lymphoblastic leukemia (ALL) is one of the most common malignancies among children. The disease is associated with excessive proliferation of lymphoid cells, as well as reduced susceptibility to programmed cell death. The blasts exhibit defective apoptosis leading to their accumulation but the mechanism of this low susceptibility to apoptosis is not fully understood. Calpains (m- and m-) are ubiquitous cysteine proteases activated by elevated cytoplasmic calcium. The role of calpains includes regulation of both proliferation and apoptosis, where they can act as either pro- and anti-apoptotic. Excessive activity of calpains is controlled by an endogenous inhibitor – calpastatin; in healthy cells calpains and calpastatin remain in a strictly regulated balance, forming the calpain-calpastatin system (CCS).

The aim of this study was to assess the potential role of CCS activity in the inhibition of apoptosis of ALL blasts and the effect of its pharmacological modulation.
Materials and methods: $CD19^+$ cells derived from bone marrow from ALL and non-oncological pediatric patients were studied. The CCS protein amounts were measured by FACS and the actual intracellular calpain activity was assessed by western blot detection of calpastatin degradation products. Apoptosis (spontaneous and Calpain Inhibitor IV – modulated) was assessed by flow cytometry, using JC-1 for detection of mitochondrial membrane depolarization and Annexin V plus 7-AAD staining for quantification of later stages of apoptosis.
Results: We have observed highly elevated amount and activity of m -calpain, whereas the level of m-calpain was low in the blasts compared to healthy B cells. The endogenous activity of calpain, measured by calpastatin degradation, was higher in the blasts, especially in the older (10^+ years) patients. Spontaneousapoptosis of the blasts was inhibited and correlated with the level and activity of m-calpain. The Calpain Inhibitor IV treatment strongly increased apoptosis.
Conclusions: The results show highly disturbed equilibrium of the enzyme and inhibitor in the CCS, which can lead to block the blast apoptosis in ALL. Calpastatin undergoes proteolytic degradation in blasts, losing ability to inhibit overactive calpain, which prevents cell death of blasts. This makes CCS a potential therapeutic target in childhood ALL.

P0729

The roles of Fas death receptor signalling and NF-kB transcription factors in autoimmune disease

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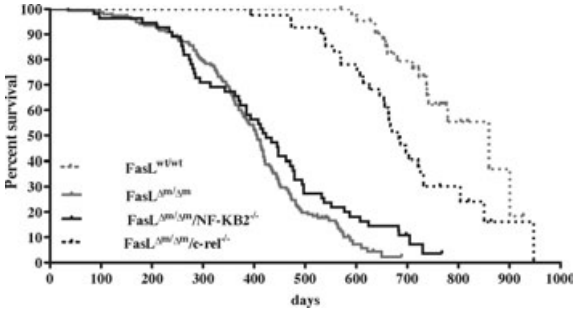
Purpose/Objective: The death receptor Fas and its ligand, FasL, are guardians against autoimmune disease and lymphadenopathy. FasL is initially produced in membrane-bound form (mFasL), which can be processed into secreted FasL (sFasL). Mice expressing only sFasL (ΔmFasL) develop glomerulonephritis and SLE-like autoimmunity or histiocytic sarcoma because membrane-bound FasL is essential for Fas-induced apoptosis. With increasing age, the ΔmFasL mutant mice accumulate abnormally increased cytokine levels (IL-6, TNF-a) and activated (nuclear) NF-κB expressing cells within inflammatory infiltrates. This is manifest before overt disease, indicating that abnormally increased NF-κB activation and increased pro-inflammatory cytokines may be critical for disease development in mice in which Fas-induced apoptosis is impaired.

Aims: Determining whether NF-kB component(s) is/are critical for the development of SLE-like autoimmune disease and tumorigenesis has the potential to identify novel therapeutic strategies. We assessed the role of different NF-κB subunits in the development of autoimmunity in the $FasL^{\Delta m/\Delta m}$ mice by crossing these animals with gene-targeted mice lacking individual NF-κB components (*nfkb2*$^{-/-}$ *and c-rel*$^{-/-}$).
Materials and methods: Development of lymphadenopathy, splenomegaly, serum Ig levels, anti-nuclear antibodies, serum cytokine levels, T regs confocal analysis of organ specific autoantibodies and pathological features associated with glomerulonephritis, vasculitis, organ inflammatory infiltrates and tumourigenesis were assessed.
Results: We report that loss of *nfkb2* did not prolong survival of $FasL^{\Delta m/\Delta m}$ mutant mice; in fact, surprisingly, the $FasL^{\Delta m/\Delta m}$;*nfkb2*$^{-/-}$ double-mutant mice developed accelerated lympho-proliferative



disease. In contrast significant reduction of autoimmune pathology and greatly prolonged survival were seen in $FasL^{\Delta m/\Delta m}$ mice ($P < 0.0001$) that lacked the classical NF-kB pathway component c-Rel.

Conclusions: NF-kB2 dependent processes must normally restrain proliferation and/or survival of excess lymphocytes that accumulate as a consequence of defects in FasL-Fas mediated killing. However therapies targeting c-Rel expression, activation or function may be promising therapeutic strategies for a range of autoimmune diseases and possibly cancers, particularly those driven (at least in part) by defects in death receptor signalling.

P0730

The soluble cytoplasmic tail of CD45 (ct-CD45) induces a non-canonical form of anergy in human T cells

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Purpose/Objective: The cytoplasmic tail of CD45 (ct-CD45) is proteolytically cleaved and released upon activation of human phagocytes. The soluble ct-CD45 was found to act on T cells as an inhibitory, cytokine-like factor that reduces T cell proliferation. In this study, we aimed to elucidate the molecular mechanisms acting within T cells, upon ct-CD45 binding.

Materials and methods: Human primary T cells were stimulated *in vitro* via plate-bound antibodies in the presence of immobilized ct-CD45. The cells were analyzed for their cytokine release and proliferative capacity. Gene expression profiling was performed via microarrays and qPCR.

Results: Here, we demonstrate that ct-CD45 induces a novel form of anergy in human peripheral blood T cells. Ct-CD45 inhibited the proliferation of purified $CD4^+$ as well as of $CD8^+$ T cells, the cytokine production (IL-4, IFN-γ, IL-10, IL-17, IL-13) and the induction of typical T cell activation markers (CD25, CD69). Co-stimulation via CD28 or CD63 failed to prevent this inhibitory signal. Moreover, we found that T cells activated via CD3/CD28 or CD3/CD63 in the presence of ct-CD45 failed to proliferate in response to restimulation which was reversible by the addition of exogenous IL-2 or IL-7. Recent studies have clearly demonstrated that such a hypo-proliferative or anergic state in T cells is not a simple loss of signaling molecules, but an active process where 'anergy factors' are being induced and synthesized to establish and maintain the unresponsive state. Classical anergy-associated genes encode, among other proteins, transcription factors (early growth response protein 2 and 3, EGR2, EGR3), E3 ubiquitin ligases (e.g. Casitas B cell lymphoma b, CBL-b), and diacylglycerol kinases (e.g. DGK-α). However, when we analyzed the gene expression profile of ct-CD45-induced anergic T cells we did not observe induction of any of these anergy factors. Characterizing the expression patterns of cell cycle regulatory factors, we found inhibition in the induction of cyclin D1 while other cyclins were unaltered.

Conclusions: Ct-CD45 triggers an anergy program in T cells which is reversible by exogenous IL-2, acting independently of classical anergy factors. From our data, the inhibition of cyclin D1 suggests a cell cycle arrest in the early G1 phase, thus making it distinct from canonical T cell anergy.

P0731

Tonic type I interferons and CD40 Signals drive breakdown of $CD8^+$ T-cell tolerance following depletion of suppressive $CD4^+$ T cells

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Purpose/Objective: Autoreactive T cells that have escaped negative selection in thymus must be controlled in the periphery to prevent autoimmunity. Under steady state conditions resting dendritic cells (DC) continously present self-antigens resulting in the deletion or inactivation of autoreactive T cells and therefore play a key role in peripheral tolerance induction. We have recently shown that cognate interactions between $CD4^+$ FoxP3 regulatory T cells (Treg) and DC are essential to maintain the tolerizing capacity of the DC *in vivo*. DC that could not interact directly with Tregs were completely unable to induce peripheral $CD8^+$ T-cell tolerance. Instead those DCs showed an activated phenotype and primed a functional cytotoxic T-cell response. The objective of this study was to identify the signals that drive the functional activation of DC in the steady state following depletion of regulatory $CD4^+$ T cells.

Materials and methods: We use DIETER transgenic mice that allow the inducible presentation of virus derived cytotoxic T cell epitopes selectively on DC. Induction of antigen presentation on steady state DC in DIETER mice results in T cell tolerance if the DC can interact with suppressive $CD4^+$ T cells but T cell priming if such suppressive interactions are not possible. We use DIETER mice in combination with mice deficient for various DC activating molecules to identify the signals that drive the functional activation of DC in the steady state following depletion of regulatory $CD4^+$ T cells.

Results: We show that the functional activation of steady-state DC and the concomitant breakdown of T cell tolerance in the absence of Treg is driven by tonic levels of Typ I Interferon and CD40 Ligation delivered by adaptive immune cells but is independent of direct recognition of pathogen associated molecules through MyD88 dependent receptors.

Conclusions: Peripheral tolerance induction by steady state DC requires regulatory T cells which counterbalance DC activation through tonic levels of Type I Interferons and CD40 signals.

P0732

Toso deficiency results in defective survival of activated effector lymphocytes

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Purpose/Objective: The regulation of lymphocyte apoptosis is critical to immune cell homeostasis and normal functioning of the adaptive immune system. Even a small imbalance in the regulation of lymphocyte survival and apoptosis can have severe pathological consequences. Recently we demonstrated that the immune-specific protein Toso represents the unique case of a cell surface molecule that protects from death receptor-induced apoptosis solely by cellular expression *(Nguyen et al., 2011)*. Here we investigated the role of Toso in survival and apoptosis of lymphocytes during cellular activation.

Materials and methods: Utilizing a genetic system of Toso-deficient mice our study followed a multidisciplinary approach, which involved biochemical, cell biological and immunological methods to analyze the impact of Toso on T and B cell function.

Results: Our studies showed that Toso is expressed exclusively in lymphoid organs, and is particularly highly expressed in T and B cells. Toso expression levels in T and B cells are regulated by cellular activation and pro-survival cytokines, such as IL-2, IL-4 and IL-15. To investigate the physiological function of Toso, we have successfully generated constitutive, as well as T and B cell specific conditional Toso knock-out mice. $Toso^{-/-}$ mice exhibited normal T and B cell development in the thymus and bone marrow, respectively, and had normal numbers of T and B cells in peripheral lymphoid organs. Functional studies showed that Toso deficiency results in increased sensitivity to TNFa-induced apoptosis and impaired survival and expansion of effector T and B cells. Furthermore, upon antigen receptor triggering $Toso^{-/-}$ B cells exhibited dramatically reduced proliferation, which was associated with a significantly higher degree of cell death. Consistent with an anti-apoptotic function of TOSO, increased apoptosis was also observed in activated $Toso^{-/-}$ T cells.

Conclusions: Given the anti-apoptotic/pro-survival function of Toso in death receptor signaling together with the remarkable upregulation of Toso expression in effector T and B cells, it is likely that Toso contributes to the generation and survival of effector T and B cells during an immune response. Thus, our studies provide important clues about the overall role of Toso in T and B cell immunity by regulating cellular apoptosis and survival of lymphocytes.

Poster session: Trafficking and Homeostasis

P0733
B cells determine T cell number and phenotype

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Purpose/Objective: While it is well established that T cells promote B cells expansion by providing 'help' during immune challenges, B cells have also been proposed to enhance or dampen T cell activation. In an attempt to clarify this controversial issue, we tested the contribution of B cells in the regulation of T cell number and phenotype at steady state and in lymphopenic conditions.
Materials and methods: Steady state analyses were performed on mice either WT or genetically deficient for B cells ($JHT^{-/-}$), secreted IgM ($\mu S^{-/-}$), switched Ig and hypermutated IgM ($AID^{-/-}$) or total Ig ($AID^{-/-}\mu S^{-/-}$) by FACS. Lymphopenia induced proliferation was monitored upon adoptive transfer of $Foxp3^-$ CD4 T cells into $TCR\beta^{-/-}$, $Rag^{-/-}$ or $TCR\beta^{-/-}$ $AID^{-/-}\mu S^{-/-}$ recipient mice.
Results: Analysis of WT animals raised in strict SPF condition revealed a positive correlation between splenic B and T cells numbers. Strikingly, while presenting up to threefold more B than age-matched WT animals $\mu S^{-/-}$, $AID^{-/-}$ and $AID^{-/-}\mu S^{-/-}$ mice maintained a similar correlation between B and T cells numbers in the spleen. Supporting further the idea that B cells control T cell numbers, mice devoid of B cells ($JHT^{-/-}$) presented a 2-fold reduction in the number of peripheral but not thymic T cells, when compared to age matched WT animals. Moreover, adoptive transfer of WT B cells corrected the T cell phenotype in $JHT^{-/-}$ mice. The reduction in T cell numbers in $JHT^{-/-}$ mice affected all subsets of T cells but was most marked for activated and Foxp3+ regulatory CD4 T cells. Adoptive transfer of $Foxp3^-$ CD4 T cells into lymphopenic mice B cell deficient ($Rag^{-/-}$) or not ($TCR\beta^{-/-}$) revealed that B cells also promote T cell recovery as well as Foxp3 induction in lymphopenic settings. This result was reproduced when using T cell less, Ig less recipient mice ($TCR\beta^{-/-}AID^{-/-}\mu S^{-/-}$), suggesting that B cell enhancing effect on T cells it is independent of the control B cells exert on the microbiota.
Conclusions: Our results indicate that B cells promote peripheral T cell expansion and/or maintenance as well as conversion to a Foxp3+ phenotype. This work should help reconsidering the contribution of B cell to disrupted homeostasis in conditions of autoimmunity, immunodeficiency and lymphopenia, the latter often induced upon chemotherapy.

P0735
Characterization of lymphocyte egress from salmonella-infected Peyer's patches

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Purpose/Objective: Lymphoid organ hypertrophy is a well acknowledged phenomenon which arises during inflammation. Here we characterize hypertrophy of intestinal Peyer's patches (PP) during local *Salmonella* infection.
Materials and methods: Using PP cell-specific labelling combined with intestinal lymph isolation, we established a method to selectively monitor lymphocyte egress from PP. We mark all PP cells *in-vivo* by FITC injection and monitor the kinetics of their replacement by incoming non-labelled lymphocytes and the dissemination of FITC-labelled cells into lymph.
Results: In *Salmonella*-infected PP, FITC-labelled lymphocytes were retained substantially longer than in non-infected PP. Moreover, the number of PP-derived cells in intestinal lymph was reduced during infection. Lymphocyte proliferation or increased recruitment did not substantially contribute to PP hypertrophy. These results reveal that infected PP establish an exit blockade, characterized by retention of incoming lymphocytes and reduced egress of PP-derived lymphocytes into intestinal lymph.

Signals initiating lymphocyte sequestration in lymphoid organs include type I interferons (IFNalpha/beta) and tumor necrosis factor (TNF)-alpha, both suggested to interfere with sphingosine-1-phosphate (S1P)-guided entry into lymph. In our model, neither type I interferon receptor (IFNAR) nor p55/TNF receptor (TNFR1) were required for lymphocyte entrapping in *Salmonella* infected PP, even though inhibition of S1P function largely blocked lymphocyte exit from PP into lymph. Moreover, lymphocyte sequestration in infected PP was independent of CD69.
Conclusions: We conclude that PP hypertrophy during infection primarily results from reduced lymphocyte egress. Moreover, our results indicate that the mechanisms which establish lymphocyte retention in infected lymphoid organs are different from those mediating transient lymphocyte retention.

P0737
Different actors, different acts, same play — plasticity of dendritic cell migration

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Purpose/Objective: Chemotaxis of peripheral dendritic cells (DCs) to secondary lymphoid tissue is an essential requirement for adaptive immunity. Recent observations suggest that migration of DCs is characterized by a high degree of plasticity depending on their own proteome and the composition of their environments. In contrast to cell migration on two-dimensional substrates, which highly depends on integrin-mediated cell attachment and the subsequent generation of intracellular force and traction, interstitial migration differs substantially. In this 3D-environment DC migration functions adhesion-independent and is predominantly based on a highly dynamic actin cytoskeleton. We are interested in defining the specific role of different molecules which regulate these distinct migration modes of DC.
Materials and methods: We employ a wide variety of methods in the field of cell biology, immunology, biochemistry and molecular biology. These state-of the art techniques include molecular genetics for manipulation of both human and murine cells, standard analytical/preparative biochemistry, highly resolved static and dynamic light microscopy and flow cytometry. Furthermore, we employ different *in vitro* cell adhesion and migration assays.
Results: Our group has shown that the tetraspanin CD81 plays a crucial role in Rac activation and the formation of actin based membrane protrusions during integrin-dependent migration on 2D-surfaces. Interestingly, we found that CD81 is dispensable for adhesion-independent DC migration in complex 3D-environments. In contrast, activity of Dynamin 2, a large GTPase which is upregulated following maturation of BMDCs, is needed for functional DC migration on 2D- and in 3D-environments, since treatment with chemical Dynamin inhibitors strongly attenuates migration of DCs in these settings.
Conclusions: DCs are able to adapt to different mechanical migration modes. Migration-modulators can either be part of the overall migration machinery, such as Dynamin 2, or they are specific regulators of distinct migration modes, like CD81 in the adhesion-dependent migration on 2D-substrates. Both the elucidation of new modulators and the subsequent characterization of the precise molecular function in DC migration could improve DC-mediated vaccination.

P0738

Lymphotoxin beta regulates lymphatic vessel expansion in mucosal ectopic lymphoneogenesis

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Purpose/Objective: Defective drainage of immune cells from inflammatory sites contributes to persistence of inflammation. Lymph nodes show synchronized expansion of the vascular and lymphatic system in response to immunization. We used a novel model of ectopic lymphoneogenesis in WT and KO mice, to evaluate whether similar events take place in the periphery and assess the modifications occurring to the vascular systems in different phases of the inflammatory process.
Materials and methods: Histological analysis in WT and LTβRKO salivary glands, cannulated with 10^{-8} p.f.u. of adenovirus, was performed at different time points post cannulation (pc). Flow cytometry was used to identify in digested tissues the CD45-EPCAM-CD31+GP38$^-$ blood endothelial cells (BEC) and CD45-EPCAM-CD31+GP38+ lymphatic endothelia cells (LEC). Immunofluorescence (IF) for CD31 and Lyve was used to validate the FACS data.
Results: By flow cytometry we observed a significant deflection in the BEC percentage of in the early phases of the inflammatory process, followed by a progressive return to resting conditions during resolution. Conversely a significant increase in the number of LEC was observed in the early phase, followed by a significant decrease ($P > 0.01$) at the peak of the inflammatory process and a drastic increase during resolution $P = 0.01$. Interestingly, while LEC expansion was due to increase in the number of small lymphatics, the second peak corresponded to the histological finding of a small number of lymphatic with an enlarged lumen. This change in number and shape of the lymphatic bed coincided with the infiltration of B-lymphocytes and peak of lymphotoxin signal in the gland. Accordingly, cannulation of LTβRKO, demonstrated absence of the second peak of LEC proliferation and lack of lymphatic expansion by IF, suggesting a role for this molecule in lymphatic modification during inflammation. LTβRKO mice did not show significant differences in the percentage of BEC as compared to the WT.
Conclusions: Ectopic lymphoid follicles associated vasculogenesis fails to recapitulate the regulation observed in secondary lymphoid organs. The changes observed in the lymphatic bed seems to be regulated by the presence of ectopically expressed lymphotoxin, suggesting a novel therapeutic role for this molecule in inflammation.

P0739

Postnatal dynamics in small intestinal immune cell populations

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Purpose/Objective: More than half of the body's professional immune cells are involved in the maintenance of intestinal homeostasis. Already in the fetus gut associated lymphoid tissues begin to form and the highly dynamic process of intestinal immune homeostasis is initiated. During delivery of the neonate a transition of the intestine from a sterile to an increasingly colonized environment occurs. The commensal flora is a very important modulator of the innate and adaptive immune mechanisms that contribute to the homeostasis. Therefore, we hypothesised that the postnatal adaptation and colonization of the small intestine directly influences the intestinal immune cell composition. The present study aims to investigate the postnatal dynamics in small intestinal immune cell populations.
Materials and methods: Small intestine immune cells were isolated and subjected to FACS analysis. The following markers of CD45+ cells were assayed to define subpopulations: CD4, CD8$\alpha\alpha$, CD8$\alpha\beta$, TCR$\alpha\beta$, TCR$\gamma\delta$, MHCII, MHCII CD11c, MHCII CD11b F480.The immune cell subsets in the small intestine of B6 mice were analysed at d6, d12, d28 and d56 after birth.
Results: During the 2nd week after birth mainly the B cell population (MHCII$^+$ CD11c-CD11b-F480-) expands and reaches a peak around day 12. TCR$\gamma\delta$ and CD8$\alpha\alpha$ subsets start to expand between d12 and day 28 after birth whereas the TCR$\alpha\beta$ and CD8$\alpha\beta$ positive population increases only after day 28. The relative abundance of the CD4 positive cells decreases from day 12 onwards. The myeloid fraction of the small intestinal immune cells (MHCII+ CD11c+ and MHCII+ CD11b+ F480+) remains quite stable over time.
Conclusions: The lymphocyte composition in the small intestine is highly dynamic whereas the relative abundance of the myeloid compartment remains stable during the postnatal period.

P0740

TCR triggering controls IL7 responsiveness and homeostatic expansion of CD4+ T cells during IL7 therapy

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Purpose/Objective: Lymphocyte regeneration following high dose chemotherapy occurs via homeostatic peripheral expansion (HPE). While HPE efficiently regenerates CD8$^+$T cells, it is inefficient for CD4$^+$ and induces a skewing of the lymphocyte repertoire towards cells with high affinity for self-peptide MHC complexes (p-MHC). Interleukin-7 (IL7) is essential for thymopoiesis and HPE of naïve T cells. Previous studies in humans have demonstrated that supraphysiological doses of IL7 could increase T cell counts and TCR diversity. However, the exact mechanism is not completely understood. Given the critical role of thymopoiesis in CD4$^+$ recovery, we hypothesized that the proliferative effect of IL-7 therapy must rely largely on recent thymic emigrants (RTE) expansion.
Materials and methods: Adoptive transfer of single positive CD4 and CD8 thymocytes and LN T cells into wt or lymphopenic hosts was used for all experiments. IL7 treatment consists of six daily doses of 10 μg.
Results: Following adoptive transfer of RTEs isolated from Rag-GFP mice, we did not find evidence that IL7 therapy induced stronger proliferation of RTEs. However, single positive CD4$^+$ thymocytes (CD4$^+_{SPT}$) were more responsive to IL7 therapy according to stat5 phosphorylation and BCL-2 induction and that lower doses of IL7 were sufficient to induce proliferation *in vivo*. We also found that CD4$^+_{SPT}$ were more sensitive to TCR signalling than RTEs and peripheral CD4$^+$ T cells (CD4$^+_{PERI}$) according to miR181a micro-RNA quantification. Despite the requirement of TCR stimulation for IL7 to induce proliferation of CD4$^+_{PERI}$ and CD4$^+_{SPT}$, residual proliferation of CD4$^+_{SPT}$ was observed upon transfer into IL7 treated MHC II$^{-/-}$ hosts. This proliferation was abrogated when IL7 therapy was delayed; suggesting that TCR triggering received inside the thymus was responsible for this effect. Increasing the number of dendritic cells by treating mice with FLT3 ligand augmented proliferation of CD4+ T cells by IL7. Finally, we found that cells undergoing proliferation during IL7 therapy were low affinity CD4$^+$ cells to p-MHC.
Conclusions: Together our data support a model wherein IL7 therapy affects CD4$^+$ T cells as they egress from the thymus and once in the periphery, these cells rapidly diminish their responsiveness to IL7. Therefore a residual thymic function is likely to augment the success of IL7 therapy in lymphopenic patients.

Poster Session: Transcription and Epigenetics

P0741

DELTA4BAFF alternative splicing is regulated by interferon-gamma AND SC35 protein

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Purpose/Objective: The B-cell activating factor (BAFF) is a potent survival factor involved in the pathogenesis of autoimmune diseases. Recently, we reported the discovery of a new transcript for BAFF, Δ4BAFF – lacking exon 4 -, which is mainly detected in autoimmune diseases and acts as a transcription factor for its own gene. However, the mechanisms implicated in Δ4BAFF induction and up-regulation are unknown. In this study we analyzed the induction and regulation of Δ4BAFF.
Materials and methods: First, to study the alternative splicing of *BAFF* exon 4, we transfected a minigene construct, centered on exon 4, into RAMOS B cells.To determine the proteins implicated in exon 4 inclusion/exclusion, we co-transfected the minigene together with each of the plasmids coding for the main splicing proteins (SC35, SRp40, SRp55, SRp20 and hnRNPA1), and the ratios between exon4 inclusion/exclusion were evaluated by RT-PCR. Second, we examined the effects of different cytokines on *Δ*4BAFF induction.
Results: RAMOS cells presented exon 4 skipping (ratio inclusion/exclusion: 6.8) after minigene transfection. Following co-transfection of the minigene with coding plasmids for splicing proteins, only the overexpression of SC35 showed effect in the splicing of exon 4, promoting exon 4 inclusion (ratio: >30).Incubation of different cell lines with several cytokines showed that IFN-$\gamma$was able to induce Δ4BAFF-transcript. Thus, after IFN-γ stimulation in the minigene model, the ratio inclusion/exclusion markedly decreased (1.5). IFN-γmodifies the balance between SC35 and another member of hnRNPs family (hnRNP C1/C2) favouring the alternative splicing of exon 4.
Conclusions: These results demonstrated that IFN-γ induces Δ4BAFF, modifying the function of SC35 protein and increasing the expression of hnRNPC1/C2. Our study provides an expanded conceptual view of BAFF gene regulation, and contributes to a better understanding of the mechanisms involved in BAFF up-regulation in autoimmunity.

P0742

Discovery of a new alternative splice variant of human Interleukin-4 and study of its functionality as a nonsense-mediated decay

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Purpose/Objective: Interleukin-4 (IL-4) is a pleiotropic cytokine regulator of immunity in health and disease states. IL-4 is a cytokine responsible of the Th2 response during infections with parasites, allergy or asthma. An alternatively spliced variant of human IL-4, known as IL-4δ2, is deleted of the second exon. IL-4δ2 can be considered as an inhibitor of IL-4 in immune response. Doing experiments on human T cells, a new alternative splice variant of IL-4 has been cloned and sequenced. In the present study, we explain the discovery of a new alternative spliced variant of IL-4 and investigate the expression of this new transcript.
Materials and methods: A bioinformatics study is conducted to investigate the presence of the alternative splice site consensus sequences. Using a transfection and a non-sens mediated decay (NMD) inhibition approach by cycloheximide (CHX), we show that this new transcript is targeted by NMD degradation.
Results: 101 base-paired (bp) of the second intron are retained between the second and the third exon. Alternative splicing consensus sequences are presents in the flanking sequences of the retention. The base-pairs retained downstream the second exon generate a frame-shiftinducing a premature stop codon (PTC) in the third exon. This PTC is located 53 bp upstream the junction between the exons 3 and 4, the last junction exon-exon. This is characteristic of a nonsense-mediated decay (NMD) construct.
Conclusions: The function of this new variant is likely to be involved in the regulation of the IL-4 expression. Thereby, we can imagine a role in regulating the Th2 response by this new mRNA.

P0743

DNA double stranded breaks enhance the transcription of AIRE target genes

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Purpose/Objective: The autoimmune regulator (AIRE*)* gene shows a predominant expression in thymus and is the major regulator of negative selection of autoreactive T-cells. Mutations in AIRE protein result in onset of autoimmune polyendocrinopathy-candidiasis-ectodermaldystrophy (APECED). One reported AIRE interacting parter is topoisomerase 2 and this interaction has been found to promote topoisomerase 2a initiated double stranded breaks. We aimed to study the possible involvement of double stranded DNA breaks in AIRE transcriptional activation of target genes in AIRE-expressing HEK293 cells after the treatment with etoposide or merbarone, the well known inhibitors of topoisomerase 2a.
Materials and methods: HEK cell line culture – AIRE inducible stable cell line was cultured in DMEM media supplemented with 10% tetracyclin negative fetal calf serum, 100 U/ml penicillin/streptomycin and 0.15 mg/ml G418. AIRE expression was induced with Doxycyline (Dox) at 2.0 μg/ml next day after plating. The protein expression was verified with western blotting.

RNA isolation and real time PCR- Total RNA was isolated from cells at different time intervals using Trizol reagent following manufacturer's protocol. Yield and purity of RNA was determined by nanodrop. Gene expression level of AIRE target gene was detected by quantitative PCR using qPCR SYBR Green Core Kit on ABI Prism 7900HT.
Results: We observed an upregulation of the AIRE target genes after the treatment with etoposide that stabilizes topoisomerase 2 to DNA and prevents religation of DNA breaks. In contrast, another topoisomerase 2 inhibitor, merbarone, which blocks topoisomerase mediated DNA cleavage did not result in up-regulation of AIRE target genes. This effect was not a consequence of variability in Aire expression levels but rather indicates the specific differencies of these two topoisomerase 2 inhibitors and a possible role of double stranded breaks in up-regulation of Aire target genes. We next examined the extent of DNA double stranded breaks under our experimental settings using BrdU TUNEL assay and FACS analysis. The analysis showed that indeed there was a significant increase in DNA double stranded breaks under the etoposide treatment compared to nontreated cells or cells that were treated with merbarone. Further, in co-immunoprecipitation experiment, we found AIRE interaction with topoisomerase 2 and this interaction increased in presence of etoposide.
Conclusions: With these results we conclude that etoposide enhances the up-regulation of AIRE target genes through the involvement of DNA double stranded breaks.

P0744

Epigenetic regulation of CTLA4 in different subpopulations of T cells

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Purpose/Objective: Cytotoxic T lymphocyte antigen 4 (CTLA4) transmits inhibitory signals to T cells and is critical for maintaining tolerance to self antigens. It is differentially expressed in T cell subpopulations. Regulatory T cells (Tregs) express CTLA4 constitutively, but in effector T cells CTLA4 expression is up-regulated in response to T cell activation. The mechanisms governing regulation of CTLA4 expression in T cell subpopulations are not entirely clear. Chromatin structure and histone modifications are important regulatory factors that affect the transcriptional status of genes. This study aims to determine if differential expression of CTLA4 is regulated through epigenetic mechanisms by comparing histone modification profiles of T cell subpopulations.
Materials and methods: Using immunomagnetic separation, naïve $CD4^+$ and $CD8^+$ T cells and $CD4^+$ $CD25^+$ Tregs were isolated from peripheral blood mononuclear cells. Purity of cells was determined by flow cytometry. Chromatin immunoprecipitation (ChIP) was performed with antibodies to various histone modifications, including histone H3 trimethylation at lysine 4 (H3K4me3) and at lysine 27 (H3K27me3). ChIP samples were analyzed by qPCR with primers covering different regions of *Ctla4*, including non-coding regulatory regions.
Results: H3K4me3 is a marker of active promoters and enhancers. We found that the promoter, gene body and distal regulatory regions were enriched for H3K4me3 in Tregs. In naïve $CD4^+$ and $CD8^+$ T cells, level of H3K4me3 was also high at the promoter and promoter proximal regions, although lower compared to Tregs. There was no marked difference in the level of the transcriptionally silenced euchromatin marker, H3K27me3 between Tregs and $CD4^+$ T cells. In $CD8^+$ T cells, however, all the studied regions were enriched for H3K27me3.
Conclusions: This bivalent chromatin pattern consisting of a repressive and an activating epigenetic marker could represent a 'transcription-ready' state for CTLA4 in naïve $CD8^+$ T cells. However, since Tregs and naïve $CD4^+$ T cells are very similar in their epigenetic profiles, it is possible that differential expression of CTLA4 in T cell subpopulations is regulated by other mechanisms as well. RNA Pol II presence together with epigenetic changes at the promoter and potential distal regulatory regions upon activation of T cells need further studies.

P0745

Evaluation of hematopoietic differentiation by expression level of key molecules

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Purpose/Objective: For a long time, hematopoiesis has been seen as a continuous restriction of cell fate potentials till the development of mature blood cells of all lineages. However, in last years several works documented unexpected plasticity of hematopoietic cells. So called lineage infidelity and/or aberrant expression of different molecules, including the key cell fate regulators, is usually observed in acute leukemia (AL). How exactly these changes affect the cell behavior in background of differentiation and whether they rise from naturally present plasticity of normal cells remains often unclear. Therefore we aimed to evaluate the simplified transcription profile of hematopoietic progenitors of different lineages in relation to the profile of their malignant counterparts as defined by flow cytometry.
Materials and methods: Using qRT-PCR we investigated mRNA expression of 90 different molecules and five control genes in sorted hematopoietic progenitors. The genes included key differentiation and proliferation regulators (e.g. EBF1, MNDA) and also molecules without known function in hematopoiesis (e.g. NFIL3, PAWR). The tested subpopulations included $CD34^+$ Lin- cells, B-lymphoid and myeloid subpopulations from bone marrow, T-lymphoid subpopulations from thymi and blasts from different AL cases (lymphoblastic, myeloblastic, AL of ambiguous lineage).
Results: The basic differences between lineages were observed in expression of well known lineage markers and regulators, e.g. CD19, PAX5, GATA3, thus forming expected clusters in clustering analysis; however, even some of these basic molecules (e.g. CD79a) presented with low aberrant expression not attributable to the contamination, observed mainly in early developmental stages. Specific transcription patterns were observed in AL of different lineages disclosing possible importance of currently poorly described genes (e.g. CCDC26). Complete changes in transcription profile towards different lineage was observed in particular stages of ambiguous lineage AL suggesting role of specific regulators (e.g. CSF3 signaling).
Conclusions: The fidelity of transcription profile of different lineages was lower than expected and in early stages comparable to AL. Our results show a need to redefine so called aberrant expression present also in normal hematopoietic progenitors.

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P0746

Patterns of DNA methylation in the T-cells and monocytes from the elderly and the young

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Purpose/Objective: During the process of aging, several changes contribute to the suppressed immune responses in the immune system of elderly individuals. Both adaptive and innate immune systems are affected. In T-cell compartment, the changes include aberrant T cell phenotypes and reduced activity. This is mainly the result of thymic involution that leads to the reduction of naïve and increase of memory and activated T cell numbers. In turn, monocytes are key regulators of the innate immune system because of their antigen presentation and phagocytic function. Although the numbers of monocytes are stable during the aging, their functional capacity to activate TLR or to express cytokines is often decreased.

DNA methylation is an epigenetic modification that plays an important role in several processes, including aging. DNA methylation occurs throughout the genome including repeats, genes and intergenic regions. CpG islands are 0.5–2 kb regions of DNA with high GC content that are often associated with promoter regions. The CpG methylation is linked with transcriptional silencing, as it can block the binding of transcription factors and recruit DNA binding repressors. In this study, we have identified genome-wide DNA methylation differences in CD4 and CD8 T cells and in monocytes of young and elderly individuals.
Materials and methods: $CD4^+$ and $CD8^+$ T cells, and $CD14^+$ monocytes were purified from the peripheral blood of four healthy young and four aged volunteers of Estonian Genome Centre using Ficoll gradient method combined with magnetic activating cell sorting (MACS) method. To assess the whole genome methylation and

expression patterns, Infinium HD Methylation Assay and HumanHT-12 v4 Expression BeadChips were used.
Results: We identified methylated CpG sites in human peripheral blood CD4 and CD8 T-cells and in monocyte cell populations. Many CpG sites were specifically methylated in T-cell or monocyte subpopulations. Importantly, we found several CpG positions that were differentially methylated in young and elderly individuals suggesting age-related methylation changes in studied cell populations. We have also analyzed the correlation of CpG methylation with nearby gene expression in corresponding cell populations.
Conclusions: Human peripheral blood $CD4^+$ and $CD8^+$ T-cell and monocyte populations have differential methylation patterns in young and elderly individuals. The differential methylation may contribute to the phenotypic and functional changes, and to suppressed activities of the immune responses during aging.

P0747

T helper 9 cell development under epigenetic control during T cell maturation

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Purpose/Objective: Histone modification patterns are key players in T helper (Th) cell regulation. However, their impact on differences in cytokine response patterns during maturation of the adaptive immune system is less understood. Here, we analyzed histone modifications in promoter regions of T-bet, GATA-3, PU.1, IRF4 and RORC in T cells of different maturation-status.
Materials and methods: Recent thymic emigrants (RTEs), matured naïve and memory CD4 T cells were isolated from cord blood of neonates or peripheral blood of adults for studying histone modification patterns and related gene and cytokine expression.
Results: The repressive histone H3 lysine 27 trimethylation (H3K27me3) dominated the PU.1 promoter in RTEs, whereas in matured naïve and further in memory T cells H3K27me3 overrepresentation was increasingly counterbalanced by permissive methylation at H3K4 and histone H3 acetylation. Notably, naïve T cells required more intense stimulation to switch the chromatin pattern in the PU.1 promoter from a repressive to a permissive state and thus to produce interleukin (IL)-9 than memory T cells. Inhibition of repressive histone methylation by the specific inhibitor, 3-deazaneplanocin A, induced Th9-specific PU.1 expression even with stimulation conditions that would normally lead only to the production of Th0 cytokines. On the other hand, prevention of histone acetylation by the histone acetyltransferase inhibitor, curcumin, diminished PU.1 expression after stimulation that would normally induce production of IL-9. Whereas RTEs differentiated into Th9 cells producing exclusively IL-9, matured naïve or memory CD4 T cells developed into effectors consisting of IL-9-producing cells as well as of cells capable of IL-4, IFN-γ, IL-5, or IL-17 co-production together with IL-9.
Conclusions: The data indicate that Th9 cell development is under stringent control during T cell life suggesting a growing contributive role of IL-9 in immune responses initiated by matured T cells.

P0748

The influence of NF-κB signaling in the conserved regulatory region of the AIRE gene

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Purpose/Objective: The *AIRE* (Autoimmune Regulator) gene is essential for the establishment of central tolerance in the thymus. In humans, mutations in the *AIRE* gene cause the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). *AIRE* regulates the promiscuous gene expression of tissue-restricted antigens (TRAs). NF-κB is essential in the transcriptional regulation of *AIRE* gene and the lack of NF-κB decreases Aire dependent TRA gene expression. Human *AIRE* and mouse *Aire* share a similar conserved sequence containing NF-κB binding sites 3 kb upstream of the transcription start site. The aim of the research was to study whether the NF-κB pathway affects AIRE expression through the upstream NF-κB binding site containing conserved sequence.
Materials and methods: We cloned the NF-κB conserved sequence upstream of different *AIRE* promoter fragments in a luciferase reporter plasmid. HEK293 cells were transfected with the reporter constructs and the NF-κB pathway was activated by TNFα or PMA/ionomycine stimulation. The effect of the NF-κB conserved sequence was assessed by the luciferase activity. In addition, we overexpressed various Rel family members and stimulated the cells with TNFα or PMA/ionomycine and the activity of the promoter constructs was measured via luminescence.
Results: Our preliminary results show that the NF-κB signaling does not activate the reporter gene expression through the conserved sequence upstream of *AIRE* promoter region. However, analysis of the *AIRE* promoter region without the NF-κB conserved region showed that gene expression was activated through the *AIRE* promoter during TNFα or PMA/ionomycine stimulation.Overexpression of Rel family members showed no significant effect on the activation of the promoter constructs with or without NF-κB conserved region.
Conclusions: In conclusion, our findings based on luciferase assays suggest that the conserved NF-κB sequence upstream of *AIRE* promoter region does not mediate activating signals, although, further analyses are required to reveal the asssociation between NF-κB signaling and the conserved NF-κB binding sites upstream of *AIRE* gene.

P0749

Transcriptional regulation of GARP expression

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Purpose/Objective: The membrane-associated molecule, glycoprotein A repetitions predominant (GARP) is highly expressed on activated regulatory $CD25^+$ CD4 T cells (Tregs), but not on resting Tregs or resting or activated $CD25^-$ CD4 T cells. The aim of this work was to delineate the transcriptional regulation of GARP gene expression.
Materials and methods: Human GARP promoter sequences were cloned into the basic pGL4.10 vector. Several CNS regions were inserted upstream of the minimal promoter of the pGL4.24 vector. Foxp3 was integrated in the mammalian expression vector pDEST12.2. The mouse T cell line, EL-4, was transfected with the promoter-reporter-constructs and stimulated with anti-CD3/anti-CD28 antibodies. In addition, cells were transfected with the Foxp3 expression vector and/or treated with retinoic acid (RA). The enhancer/attenuater function of CNS on the promoter activity was analyzed after transfection of CNS-reporter-constructs and stimulation with PMA and ionomycin. Chromatin immunoprecipitation of freshly isolated and activated human $CD4^+$ $CD25^+$ and $CD25^-$ T cells was performed to evaluate the trimethylation state of histone 3 at lysine 4 and at lysine 27, as well as the acetylation state of histone 3 of promoter and CNS regions.
Results: Foxp3 and RA synergistically induced luciferase expression in a concentration dependent manner. Histone modifications at the promoter region changed in $CD25^+$ Tregs towards a more accessible chromatin configuration upon T cell activation. In contrast, in $CD25^-$ T cells it was rather repressively modified. A CNS region located upstream of the GARP gene exhibited an open chromatin configuration in $CD25^+$ T cells upon TCR stimulation. When inserted upstream of a minimal promoter, this CNS region enhanced luciferase activity in response to T cell stimulation.
Conclusions: Thus, GARP transcription appears to be initiated by coordinate action of Foxp3 and RA and is additionally modulated via CNS. The transcriptionally active status of the GARP gene is supported by histone modifications in the promoter and conserved regions.

Poster Session: Asthma/Allergy

P0750

A fusion protein of flagellin and ovalbumin as novel vaccine candidate for allergies: suppressing TH2 responses and preventing murine intestinal allergy

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Purpose/Objective: The TLR5 agonist flagellin has been associated with immune modulatory functions making it an interesting adjuvant for allergy treatment. In this study we investigated the potency of fusion proteins containing *Listeria monocytogenesis*-derived flagellin flaA to induce DC activation and modulate ovalbumin-specific T cell responses. Moreover, we checked the fusion proteins potency for the treatment of allergies *in vivo*.
Materials and methods: Bone marrow-derived myeloid dendritic cells (mDC) from BALB/c, C57BL/6, IL-$10^{-/-}$, and TLR-signalling deficient ($MyD88^{-/-}$) mice were stimulated with rOva, rflaA, rflaA plus rOva, or a recombinant fusion protein consisting of rflaA and rOva (rflaA:Ova). Immune modulating properties of rflaA plus rOva and rflaA:Ova were investigated by DC:T cell co-culture using $CD4^+$ T cells from Ova-TCR transgenic or Ova/alum-immunized mice. A murine model of Ova-induced intestinal allergy was applied to assess the capacity of flaA:Ova as potential allergy vaccine.
Results: rflaA:Ova induced up-regulation of TLR5 on mDC and, dose-dependently, even higher IL-6 and IL-10 secretion than equimolar amounts of rflaA. Moreover, rflaA:Ova induced IL-10 mediated suppression of TH1 and TH2 cytokine secretion from Ova-TCR transgenic $CD4^+$ T cells and $CD4^+$ T cells derived from T_H2-biased mice upon Ova/alum immunization. The strong activation of mDC by rflaA:Ova could be suppressed in a dose-dependent manner by application of both inflammasome (Z-VAD-FMK, glybenclamid) and proteasome (lactacysteine) inhibitors. Prophylactic vaccination with rflaA:Ova was shown to protect against intestinal allergy, reduce T cell activation and T_H2 cytokine secretion. Additionally, rflaA:Ova suppressed Ova-specific IgE and induced Ova-specific IgG2a antibodies, whereas rflaA or rOva provided alone or as a mixture did not have comparable protective effects.
Conclusions: The rflaA:Ova fusion protein showed enhanced TLR-mediated immune modulating capacities, probably attributed to the proximity of the adjuvant and allergen. rflaA:Ova was able to suppress TH2 responses both *in vitro* and *in vivo*. Therefore, rflaA: allergen fusion proteins are promising vaccine candidates for intervention in IgE-mediated allergy.

P0751

A hypoallergenic variant of the major birch pollen allergen Bet v 1 shows distinct characteristics in antigen processing and T cell activation

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Purpose/Objective: BM4 is a novel genetically engineered variant of Bet v 1, the major birch pollen allergen. BM4 differs from Bet v 1 in only five amino acids but lacks the typical Bet v 1-like fold. As a consequence, BM4 displays negligible IgE-binding while it contains all relevant T cell epitopes of Bet v 1 except one. The aim of this study was to compare BM4 and Bet v 1 with regard to internalization, antigen-processing and presentation by antigen-presenting cells as well as T cell activation.
Materials and methods: Proliferative responses to BM4 and Bet v 1 of peripheral blood mononuclear cells and Bet v 1-specific T cell clones were analysed. Fluorescently labelled BM4 and Bet v 1 were used to study surface binding, endocytosis and intracellular degradation by monocyte-derived DC (mdDC). Both proteins were digested by endolysosomal extracts of mdDC. BM4- and Bet v 1-pulsed mdDC were employed to assess the kinetics of activation of Bet v 1-specific T cell clones and the polarization of naïve T cells.
Results: BM4 displayed a significantly stronger T cell-activating capacity than Bet v 1. Furthermore, BM4 bound more efficiently to the surface of mdDC. Internalisation and trafficking into acidic compartments as well as lysosomal degradation were faster for BM4 than for Bet v 1. BM4-pulsed mdDC induced enhanced proliferative responses at earlier time-points in Bet v 1-specific T cell clones. T cells primed with BM4-pulsed mdDC synthesized less IL-5 than those primed with Bet v 1-pulsed mdDC.
Conclusions: The loss of the Bet v 1-fold changes the protein's interaction with the human immune system at the level of antigen-presenting cells resulting in altered T cell responses. By combining low IgE-binding with a strong T cell-activating capacity that modulates the allergen-specific T cell response, BM4 represents a highly interesting candidate for specific immunotherapy of birch pollen allergy.

P0753

A peptide mimicking an important carbohydrate epitope cross-reactive between birch proteins and celery allergen Api g 5

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Purpose/Objective: Allergic reactions to celery are often caused by cross-reactivity to major birch pollen allergens like Bet v 1 and Bet v 2. Nevertheless, about 40% of the cross-reacting IgEs are directed towards yet unidentified allergens of 32–63 kDa in birch, and towards Api g 5 in celery. It has been demonstrated that IgE-binding to Api g 5 is strongly dependent on its carbohydrate moieties. Monoclonal antibody BIP 3 was originally raised against birch pollen extract and cross-reacts with Api g 5. For epitope definition, we aimed to generate peptides mimicking the BIP 3 epitope, so called mimotopes.
Materials and methods: Phages from a random-peptide phage-display library were selected for specific binding to monoclonal antibody BIP 3 and for their ability to inhibit IgE-binding of allergic patients to birch pollen extract. Three phage clones were chosen for intraperitoneal immunization of BALB/c mice and induced antibodies were tested on blotted birch pollen extract. To test if the mimicked epitope was comprised of carbohydrate moieties, we compared serum reactivity of immunized mice to Api g 5 and the structurally unrelated plant glycoprotein horse radish peroxidase (HRP). As a negative

control nonglycosylated grass pollen allergen Phl p 5 was used in ELISA.
Results: After 3 rounds of biopanning 7 different sequences were derived from 14 positive phage clones. The most frequent sequence also elicited the strongest immune reaction in mice. All 3 clones selected for immunization induced antibodies crossreactive for the major 63 kDa band recognized by BIP 3 in birch pollen extract and Api g 5. The antibodies induced by the mimotope immunizations were also reactive towards natural purified Api g 5 and the glycoprotein HRP, but not to the control allergen Phl p 5.
Conclusions: The peptide mimotopes characterized in this study mimic a carbohydrate epitope cross-reactive between Api g 5 and high molecular weight birch pollen proteins. As a peptide mimotope can be considered a superior immunogen in comparison to carbohydrate moieties, it may be suitable for epitope-specific immunotherapy and diagnosis.

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P0754

A pollen hybrid molecule as novel therapeutic for the treatment of Fagales multi-sensitization

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Purpose/Objective: Allergies against birch pollen and pollen of related trees belonging to the botanical order of *Fagales* are the most common cause for spring pollinosis in the temperate climate zone of the Northern hemisphere. However, specific immunotherapy is mainly performed with birch pollen extracts, limiting the success of this therapeutic intervention in areas where birch is not endemic.
Materials and methods: Thus, in the present study we generated a pollen hybrid molecule (PH) by PCR-based recombination of T cell epitopes of the low IgE-binding isoforms of the major allergens from birch, hazel, alder, oak, and hornbeam. *In silico* analyses of PH revealed that the molecule carries an epitope previously identified as critical for the Bet v 1-fold and closely connected to IgE binding, immunogenicity, and T cell polarization of Bet v 1. Therefore, the structure of PH was modified accordingly by replacing seven consecutive amino acids within the Bet v 1-part of the molecule by the homologous sequence of Mal d 1, resulting in a fold-variant of the molecule (PH4).
Results: Both hybrid molecules showed reduced binding of Bet v 1-specific IgE antibodies and an elevated activation of PBMCs from birch pollen allergic donors. Compared to the parental allergens, the pollen hybrid molecules could induce antigen-specific T_H1 cells in a mouse immunization model. In dendritic cells the modified structures of both hybrids directly affected antigen-uptake and processing. The prophylactic potential of both proteins was tested in a mouse model of oral tolerance, indicating that the pollen hybrids could protect mice from *Fagales* pollen induced lung inflammation.
Conclusions: Compared to wild-type allergens the pollen hybrid molecules interacted differently with dendritic cells, leading to a deviated T cell response *in vivo*. In combination with the low IgE binding properties our immunological data emphasizes the potential of PH and PH4, respectively, as novel tools for the treatment of *Fagales* multi-sensitization.

P0756

Absence of Foxp3+ regulatory T cells during allergen provocation does not exacerbate murine allergic airway inflammation

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Purpose/Objective: Tregs have been implicated in modulating allergic immune responses. However, their influence on distinct phases of development of allergies remains unclear. In this study we aimed to comprehend the specific involvement of $Foxp3^+$ Tregs in airway inflammation during allergen provocation. This reflects a clinically relevant situation, assuming prior allergen sensitization to have already occurred, and thus addresses a potential Treg mediated therapeutic role.
Materials and methods: Standard OVA-Alum model was used for induction of allergic airway inflammation in Foxp3-eGFP-DTR transgenic (DEREG) mice. A group of sensitized mice received diphtheria toxin treatment to deplete Tregs prior to allergen challenge. Serum immunoglobulin levels were determined by ELISA to confirm comparable sensitization across groups. Inflammation was scored by differential cellular infiltration in broncheo-alveolar lavage (BAL) and cytokines secretion by *in-vitro* re-stimulated mediastinal lymph node cells. Lung pathology was assessed by histology and qPCR.
Results: Comparable antigen-specific serum immunoglobulin levels implicate equivalent sensitization across all groups of mice. No significant alteration was observed in numbers or types of cells infiltrating BAL of Treg depleted mice. Th2 cytokines secreted by re-stimulated mediastinal lymph node cells were comparable. Double blinded histological analysis of lung sections revealed no enhanced pathology in lungs of Treg depleted mice. To rule out strain specific differences we followed similar regimen in DEREG mice on a comparatively allergy resistant genetic background (C57BL/6). Consequently, Treg depletion during allergen challenge did not enhance lung inflammation even in C57BL/6 mice.
Conclusions: Absence of $Foxp3^+$ Tregs during allergen challenge does not exacerbate allergic airway inflammation in mice. Differential disposition to allergies due to genetic backgrounds does not alter the inflammatory response observed in this regimen. We propose less pronounced role of $Foxp3^+$ Tregs under physiological conditions in inhibiting allergic responses during the allergen provocation phase as compared to their influence during the sensitization phase. These results signify the temporal regulation exerted by $Foxp3^+$ Tregs and may influence their application as potential therapeutics.

P0758

Acrolein blocks antibody-formation upon nasal sensitization with KLH in BALB/c mice

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Purpose/Objective: Smokers have an increased risk for respiratory diseases. Hence, we sought to investigate the contribution of acrolein during nasal sensitization, since acrolein is generated in large amounts in cigarettes.
Materials and methods: BALB/c mice were nasally sensitized 6 times in biweekly intervals with KLH alone or with KLH in conjunction with acrolein. Immune response was analyzed on the level of specific antibodies in ELISA and by cytokine determination from splenocytes after antigen-specific -stimulation.

Results: In the absence of adjuvant, nasal application of KLH alone was sufficient to induce KLH-specific antibody-titers of IgG1, IgG2a, IgG2b, IgA and IgE, whereas nasal sensitization with KLH in the presence of acrolein completely abrogated antibody-formation. In contrast, KLH-stimulated splenocytes of mice sensitized with KLH in conjunction with acrolein secreted higher levels of IFN-g than mice sensitized with KLH alone.
Conclusions: Nasal application of acrolein impairs the induction of a proper humoral immune response by preventing antigen-specific antibody formation, but it supports innate release of IFN-γ. Hence, acrolein in smoke significantly modulates the innate and adaptive immune responses in smokers, thereby likely affecting susceptibility to infections in smokers.

P0759

Allergen-specific immunotherapy induces regulatory sialylated IgGs

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Purpose/Objective: Allergen-specific immunotherapy (SIT; hyposensitization) is associated with the development of regulatory T cells and allergen-specific IgG serum antibodies. However, the function of SIT-induced IgG is still unclear. Recently, it has been shown that the Fc glycosylation pattern determines the pro- or anti-inflammatory effector function of IgG antibodies, whereby sialylated IgGs are anti-inflammatory. However, the IgG Fc glycosylation pattern induced by SIT is unknown. We sought to examine the Fc glycosylation and anti-inflammatory quality of IgG molecules formed upon allergen-specific tolerance induction.
Materials and methods: We administered chicken ovalbumin (OVA) under inflammatory or tolerance conditions to mice and analyzed OVA-reactive IgG Fc glycosylation. The anti-inflammatory function of differentially glycosylated anti-OVA IgGs was further investigated by studies with dendritic cell (DC) cultures and in an *in vivo* model of allergic airway disease. Additionally, we analyzed the Fc glycosylation pattern of birch pollen-reactive serum IgGs following successful SIT in patients.
Results: Stimulation with protein antigens/allergens under inflammatory conditions induced de-sialylated IgGs. In contrast, tolerance induced immunosuppressive sialylated IgGs that were sufficient to block antigen-specific T and B cell responses, DC maturation and allergic airway inflammation. Importantly, successful SIT in allergic patients also induced sialylated allergen-specific IgGs.
Conclusions: Our data show a novel antigen-specific immunoregulatory mechanism mediated by anti-inflammatory sialylated IgGs that are formed upon tolerance induction. These findings may help to understand und to develop novel allergen-specific therapies for the treatment of allergy.

P0760

Allergen-specific mucosal IgA induced following sublingual immunotherapy protect against allergic inflammation in a murine model of allergic inflammation

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Purpose/Objective: Sublingual immunotherapy (SLIT) with allergen extract has been shown to be efficacious in treating allergy. A hallmark of allergen-specific immunotherapy is the induction of non-IgE allergen-specific antibodies primarily of the IgG1 and IgG4 isotypes which may, in addition to being robust markers of immunotherapy, be at least partly responsible for the clinical effect of the treatment through their capacity to compete with effector-cell bound IgE antibodies for allergen binding. It has also been hypothesized that allergen-specific IgA antibodies induced after successful SLIT could act in a similar manner and prevent allergen absorbance, and thereby subsequent allergic reactions. The objective of this study was to use a knock-out mouse strain lacking active transport mechanisms of IgA to mucosal surfaces for exploring the role of SLIT-induced mucosal IgA in protection against allergic inflammation.
Materials and methods: Wild type (WT) and poly immunoglobulin receptor knock-out (pIgRKO) mice were sensitized to *Phleum pratense* (Phl p) by intraperitoneal injections of alum-adsorbed allergen extract. Sensitized mice were administered Phl p extract or buffer sublingually. Thereafter, mice were challenged intra-nasally with Phl p pollen in order to mimic a grass pollen season. Numbers of sneezes, allergen-specific antibody levels and total numbers of eosinophils in nasal lavage (NAL) and bronco-alveolar lavage (BAL) were used as read-outs for the efficacy of SLIT. Moreover, the induction of allergen-specific IgA on mucosal surfaces and in serum was determined following 9 weeks of sublingual administration.
Results: SLIT with Phl p extract in WT mice significantly suppresses allergen-induced inflammation measured as attraction of eosinophils to BAL and induction of allergen-specific IgE antibody response in BAL and serum. This protection following Phl p SLIT towards allergen-induced inflammation could not be observed in the pIgRKO mice lacking active transport of IgA to mucosal surfaces. In addition, we could find higher allergen-specific IgA response in saliva, serum and BAL from WT mice as compared to pIgRKO mice.
Conclusions: Allergen-specific mucosal IgA antibodies are induced after SLIT in WT mice and seem to play a protective role against allergic inflammation in a murine model of grass pollen induced allergic rhinitis.

P0761

Allergen–specific T and B cell proliferation in pollen allergic patients measured by a CFSE dilution based assay

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Purpose/Objective: In past years alternative methods to radioactive measurements for cellular proliferation, such as assessment of dilution of the dye 5, 6-carboxy-fluorescein diacetate succinimidyl ester (CFSE) in the flow cytometer, have been established. The latter method has one major advantage: It allows not only for measurement of proliferation in total peripheral blood mononuclear cells (PBMCs) but also in subpopulations e.g. T cells or B cells by multicolour flow cytometry. In the present study we sought to establish a CFSE dilution based assay to determine which cell types contribute to a proliferative response to allergen *in vitro*.
Materials and methods: To that aim we isolated PBMCs from birch and grass pollen allergic patients and labelled them with CFSE. Cells were cultured in the presence or absence of highly purified recombinant allergens (rBet v 1, rPhl p 5) for 1 week whereupon proliferation was measured by flow cytometry.
Results: T cell proliferation upon allergen stimulation was observed in PBMC cultures of all patients over a wide range of allergen concentrations using co-staining for CFSE and the pan T cell marker CD3. More importantly co-staining for the B cell marker CD20 revealed, that also a subfraction of B cells proliferated in response to allergen stimulation.

Conclusions: Thus we demonstrated that in PBMC cultures of allergic patients both B and T cells proliferate specifically in response to allergen stimulation *in vitro*. Since each of the tested patients mounted allergen-specific antibody responses it is possible that the proliferating B cell sub-fractions contained allergen-specific B cells. The fact that in PBMC cultures of allergic patients not only T cells proliferate in response to allergen should be borne in mind when interpreting classical proliferation assays such as ^{3}H thymidine incorporation.

P0763

Alpha purothionin, a new wheat food allergen, belongs to a family of plant defence proteins

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Purpose/Objective: Wheat is an important source for IgE-mediated food allergy. Avoidance of wheat products is currently the only therapy for wheat food allergic patients whereas allergen-specific approaches such as immunotherapy would require a detailed knowledge and availability of the disease-causing allergens. Aim of this study was the isolation, identification and characterization of new allergens recognized by wheat food allergic patients for diagnosis and treatment of wheat food allergy.
Materials and methods: We screened a wheat cDNA library with serum IgE antibodies from patients suffering from wheat food allergy. The cDNA coding for novel wheat food allergen, alpha-purothionin, could be isolated and identified by sequence analysis. Recombinant alpha-purothionin was expressed, purified and characterized regarding molecular properties. The IgE-reactivity was tested in wheat food allergics, grass pollen allergic patients and non-atopic individuals. Allergen-specific rabbit antibodies were used to screen different cereal and bread extracts. To investigate the allergenic activity, we performed basophil degranulation experiments.
Results: In this study we report the isolation of an IgE-reactive cDNA clone coding for a novel wheat food allergen alpha purothionin which belongs to a family of plant defence proteins. Homologue proteins to alpha purothionin could be detected by allergen specific rabbit antibodies in many other cereal and bread extracts. Serum IgE antibodies from wheat food allergic patients reacted specifically with alpha purothionin and allergenic activity was demonstrated in basophil degranulation and skin prick test experiments.
Conclusions: Recombinant alpha purothionin may be useful for the diagnosis and possibly immunotherapy of IgE-mediated wheat food allergy.

P0764

Analysis of the lung inflammation induced by Alternaria alternata spores or extracts in C57BL/6, TLR-4 and MyD88 knockout mice

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Purpose/Objective: The mould *Alternaria alternata* is an important cause of allergic rhinitis and asthma and exposure to airborne spores of *Alternaria* can trigger severe asthma. Our objective was to analyse the lung immune response induced in mice instilled with spores or extracts of *Alternaria* and to analyze the requirement of TLR4 and MyD88 signalling for its development.
Materials and methods: WT C57BL6, TLR4 KO or MyD88 KO mice were instilled with *Alternaria* spores or extracts. Cellular recruitment in the lungs was analysed early (3 days) or later (5 weeks) during the response. Cytokine/chemokine lung expression profiles were investigated at the same time points by RT-qPCR.
Results: After 3 days, C57BL/6 mice showed an accumulation of neutrophils in the airways, this recruitment was the strongest with the spores. This stronger inflammatory property was confirmed by a stronger activation of IL-17, CXCL-2, CCL-3 and IL-1a expression in the lungs of mice stimulated with the spores. After 5 weeks a mixed lung inflammatory response was induced by the spores. In contrast, only eosinophils were recruited after extract instillation. This correlated with an increased lung expression of IL-4, IL-13, CCL11 and CCL24. Of note, the expression of IL-33 was similar for spores and extracts at week 5 but only the extract induced an early expression of this cytokine at day 3. Very similar cell recruitments were observed in wild type C57BL/6 mice and in TLR-4 KO mice stimulated with the extracts. However mice genetically inactivated in the MyD88 adaptor had an almost complete inhibition of lung neutrophil recruitment at day 3 and eosinophils at week 5. This correlated with a decreased expression of inflammatory gene expression, an inhibition of IL-33 expression at day 3 and a decreased IL-4, IL-13, CCL11 and CCL24 expression at week 5.
Conclusions: *Alternaria* spores and extracts have inflammatory and allergic properties, be it that spores have a tendency to induce inflammatory responses while extracts induce stronger allergic responses. TLR-4 signalling is dispensable but MyD88 is compulsory for these responses. Moreover, there is a correlation between an early IL-33 expression, a low inflammatory response and the accumulation of eosinophils during the chronic phase induced by the extract.

P0766

Apoptotic effect of Tamoxifen on neutrophils bronchial cells in horses with Recurrent Airway Obstruction: a new therapeutic approach

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Purpose/Objective: Recurrent airway obstruction (RAO, 'heaves') is an asthma-like condition that develops in mature horses following stabling and exposure to dusty hay and straw. The hallmark of this disease is that hay/straw exposure induces clinical airway obstruction, airway neutrophilia and increased airway mucous production in RAO susceptible horses. The aim of this work was to evaluate the rate of apoptosis in bronchial neutrophils in RAO-affected horses during acute crisis and remission; to observe the effect of the treatment on the

apoptosis rate of affected horses with tamoxifen; and to evaluate the concordance of these findings with clinical signs, levels of specific immunoglobulin and type of cells found in bronchoalveolar lavage fluid (BALF).

Materials and methods: For this purpose, nine RAO susceptible horses sensitized to *Aspergillus fumigatus* (RAO herd) were selected for use in this study. The animals will be exposed to dusty/moldy hay, and once the signs of the disease appear were carefully evaluated recording clinical signs, result of endoscopic examination; and BALF evaluation (immunoglobulin and cellular content). Later, will be treated with 100 mg PO of tamoxifen every other day in a remission environment. During treatment and after the drug administration ended, periodical evolution of RAO signology. The sampling will include physical and endoscopic examination and collection of BALF and blood for analysis. Flow cytometry using commercial AnnexinV-FITC and propidium iodide was used to quantify early and late apoptotic leukocytes, respectively.

Results: The results showed a significant increase in early apoptosis in peripheral blood and bronchial granulocytic cells in RAO-affected horse's treatred with tamoxifen. In addition, we showed that RAO-affected horses treated with this drug displayed a significant decrease in neutrophils in BALF and a concomitant improvement in their clinical status; with a decrease in total levels of immunoglobulins against *A. fumigatus*.

Conclusions: Our study showed that tamoxifen, a drug used for breast cancer, has the ability to induce apoptosis in granulocytic cells from peripheral blood and BALF with a concomitant improvement in their clinical status. Finally, further *in vitro* and *in vivo* studies are needed to better understand tamoxifen treatment because as an anti-carcinogen, tamoxifen could be used to treat chronic, inflammatory pathologies including those associated with granulocytes and allergic diseases, such as asthma or equine RAO

P0767

Association of HLA-DR1 with the allergic response to the major mugwort pollen allergen: molecular background

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Purpose/Objective: Mugwort pollen allergens represent the main cause of pollinosis in late summer. The major allergen, Art v 1, contains only one single immunodominant, solely HLA-DR-restricted T cell epitope (Art v 1_{25-36}). The frequency of HLA-DRB1-01 is highly increased in mugwort-allergic individuals and HLA-DR1 serves as restriction element for Art v 1_{25-36}. However, Art v 1_{25-36} also binds to HLA-DR4 with high affinity and DR1-restricted Art v 1_{25-36} -specific T cell receptors can be activated by HLA-DR4 molecules.

Materials and methods: To understand the predominance of HLA-DR1 in mugwort allergy in spite of the degeneracy in HLA/peptide-binding and TCR-recognition, we investigated the molecular background of Art v 1_{25-36}/MHC/TCR interactions in the context of HLA-DR1 compared to -DR4.

Results: The majority of Art v 1_{25-36} -specific T cell lines and clones from HLA-DR1 carrying, mugwort pollen-allergic donors reacted to synthetic and naturally processed Art v 1 – peptides when presented by HLA-DR1 or HLA-DR4 expressing antigen presenting cells. However, at limiting peptide concentrations DR1 was more effective in T cell stimulation. In addition, the minimal epitope for 50% of Art v 1_{25-36} – specific T cells was shorter for DR1 than for DR4. *In vitro* binding assays of Art v 1_{25-36} mutant peptides to isolated DR1- and DR4-molecules indicated similar binding capacities and use of the same register. In silico simulation of Art v 1_{25-36} binding to HLA-DR1 and -DR4 suggested similar binding of the central part of the peptide to either molecule, but a higher flexibility of the N- and C-terminal amino acids and detachment at the C-terminus in HLA-DR1.

Conclusions: The predominance of HLA-DR1 in the response to Art v 1_{25-36} may be explained by the tendency of superior peptide presentation by DR1 compared to DR4 found *in vitro*. Computer simulation supported our experimental data by demonstrating differences in peptide mobility within the HLA-DR complex which may influence TCR-binding. We suggest that the minor differences observed *in vitro* may be more relevant in the microenvironment *in vivo*, so that only presentation by HLA-DR1, but not -DR4 permits successful T cell activation.

P0768

Bone marrow-derived dendritic cells inappropriately reflect allergen sensitization with house dust mite aeroallergens in the context of complement activation

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Purpose/Objective: Pulmonary dendritic cells play critical roles in allergen uptake and Th cell differentiation towards Th2 and Th17 effector cells. Previous studies have demon-strated a protective role for C5a and a proallergic role for C3a during allergen sensi-tization suggesting that C3a and C5a either promote or suppress DC functions during initial allergen encounter. Using bone-marrow-derived (BM) DCs as a surrogate for pulmonary APCs, we aimed at better defining the roles of C3a and C5a in DC-mediated development of maladaptive Th2 and Th17 immunity in allergic asthma.

Materials and methods: GM-CSF-differentiated bone marrow BMDC from wildtype (wt), $C3aR^{-/-}$, $C5aR^{-/-}$ and $C3aR^{-/-}$/$C5aR^{-/-}$ mice were obtained after 9 days and were pulsed *in vitro* for 24 h with crude extract from house dust mite (HDM). 1×10^6unpulsed or pulsed cells were then transferred intra-tracheally into wt recipient mice. The following parameters were used to define the allergic phenotype: airway hyperresponsiveness (AHR), inflammatory cell infiltration, *ex vivo* cytokine production from pulmonary lung cells, histologic examination of mucus production.

Results: We found that BMDCs from wt and $C3aR^{-/-}$ mice induced a strong asthmatic response, characterized by a marked increase in AHR, strong Th2 but minor Th17 and Th1 cytokine production and a mixed eosinophilic and neutrophilic infiltration of the lung. In contrast, AHR, Th2 cytokine production and eosinophilic inflammation were substantially decreased following adoptive transfer of $C5aR^{-/-}$ or $C3aR^{-/-}$ $C5aR^{-/-}$ BMDCs. However, mice treated with $C3aR^{-/-}$ $C5aR^{-/-}$ BMDCs showed strong neutrophilic infiltration and an increased IFN-g production.

Conclusions: We found that adoptive transfer of allergen-pulsed $C5aR^{-/-}$ or $C3aR^{-/-}$ BMDCs does not recapitulate the findings of an increased allergic phenotype in $C5aR^{-/-}$ mice and a decreased allergic phenotype in $C3aR^{-/-}$ mice after intratracheal HDM administration. In the BMDC adoptive transfer setting, C5aR plays a critical role in the development of asthma whereas the role of C3aR is at best minor. Our data suggest that adoptive transfer of allergen-pulsed BMDC does not appropriately reflect the sensitization process towards aeroallergens such as HDM by lung resident DCs, at least in the context of complement activation.

P0769

Caffeic acid decreases eosinophilic airway inflammation on blomia tropicalis murine model of allergy

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Purpose/Objective: To evaluate the anti-allergic effect of Caffeic Acid (CA) in a murine model of allergy to *Blomia tropicalis* mite.
Materials and methods: Groups of 6 AJ mice were sensitized by subcutaneous injections with 100 μg of *B. tropicalis* antigen in 4 mg/ml aluminum hydroxide on days 0 and 7. Twenty-four hours (24 h) after the last sensitization the animals received four (4) intranasal challenges (10 μg per animal) at intervals of 1 day. Allergic animals were treated orally with 10, 100 or 200 mg/kg of CA or with 3 mg/kg of Dexametazone (Dex). On day 15 the animals were euthanized and some parameters were evaluated such as number of leukocytes/eosinophils in bronchoalveolar lavage (BAL), determination of eosinophil peroxidase activity (EPO) in BAL and levels of IgE anti-Bt in the serum.
Results: Treatment of animals with CA demonstrates the ability of CA in reducing allergic parameters such as the total number of cells andnumber the eosinophils in BAL ($P < 0.01$) in relation to the untreated BT-sensibilized and challenged mice. The oral treatment with CA also displayed a significant reduction in the levels of EPO in BAL ($P < 0.001$). As expected, the administration of 3 mg/kg of Dex significantly suppressed the number of eosinophils and total inflammatory cells ($P < 0.001$) and decreased EPO activity in BAL ($P < 0.001$). Bt-immunized mice produced higher levels of specific IgE antibodies than the normal control group ($P < 0.001$). However, treatment with CA was not able to reduce IgE antibody levels, in contrast to animals treated with Dex ($P < 0.05$).
Conclusions: CA effectively reduced some immunological parameters related to cell migration responsible for airway inflammation in a murine model of allergy induced by *B. tropicalis* but did not interfere in IgE antibody production.

P0770

Can innate inflammatory factors break immunotherapy-induced T cell tolerance in patients with allergic asthma?

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Purpose/Objective: Asthma is a disease of the respiratory system, with 3 main subgroups; the allergic group forming the biggest proportion. Allergen-specific immunotherapy cures allergic diseases, with great success. The immunologic mechanisms and contributing factors of immunotherapy is still a question to be answered. Innate inflammatory cytokines and ligands for certain Toll like receptors (TLRs) are shown to release T-cell unresponsiveness to allergens in PBMCs of healthy individuals, which may resemble convertion of healthy status to allergic.
Materials and methods: This study aims to investigate roles of these inflammatory factors in immunotherapy model which provides allergen-specific and induced unresponsiveness in allergic asthmatic patients. PBMCs isolated from 3 allergic asthma patients who received immunotherapy to known allergens were stained with CFSE in order to investigate allergen-specific $CD4^+$ T cell proliferation, cultured with the existence and absence of immunotherapy allergen as well as control allergen, and stimulated with IL-1beta, IL-6 and ligands for TLR4 and TLR8.
Results: First results of this ongoing study support the tolerance-breaking effects of IL-1beta and IL-6.
Conclusions: More number of patients should be investigated in order to reveal the possible roles of these inflammatory conditions in breaking of peripheral tolerance

P0771

CD4+ CD25+ FOXP3+ T cells are Decreased in Patients with Allergic Conjunctivitis

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Purpose/Objective: Allergic conjunctivitis (AC) is one of the most common eye disorders in clinical practice. It has been shown that AC is a disorder mediated by Th2 lymphocytes producing IL-4 and IL-5, where the eye damage is caused by a type I hypersensitivity. On the other hand, It has been suggested in asthma and rhinitis that T regulatory cells (Tregs) $CD4^+$ $CD25^+$ $FOXP3^+$ have been involved in control allergic status, favoring an optimal microenvironment with immunosuppressive cytokines (IL-10, TGF-β). Meanwhile the immune status of the ocular microenvironment has evolutionally adapted itself to prevent the induction of excess inflammation, thereby protecting its delicate structures from the damages of inflammation. However, it is not been established whether Treg cells can modulate allergic response. Based on the above background, the present study focused to observe the role of Treg cells in human allergic conjunctivitis.
Materials and methods: Peripheral blood mononuclear cells (PBMC) were isolated from blood samples of healthy donors (HD) and AC-patients, and then PBMC were labeled with mAbs against CD4, CD25 and FOXP3. Also PBMC were co-cultured with their autologous monocytes. After 24 h, the culture medium was removed and optimal dose of antigen stimulation *Dermatophagoides pteronyssinus* (*Der p*) was added. After 7 days, all cells were labeled with mAbs against CD4, CD25 and FOXP3. The cells labeled were analyzed by flow cytometry. The supernatant culture was recovered to analyze by ELISA the cytokines IL-10 and TGF-β.
Results: AC-patients showed 55-times more $CD4^+$ $CD25^+$ cells than HD. Most of $CD4^+$ $CD25^+$ cells were $FOXP3^-$, when we compared mean fluorescence intensity (MFI) of FOXP3 in $CD4^+$ $CD25^+$ cells, we observed a decreased expression in AC-patients than HD. We note significant increase in $CD25^+$, $CD4^+$ $CD25^+$ after antigen stimulation. However, no changes were detected in frequency of $CD4^+$ $CD25^+$ $FOXP3^+$ and $FOXP3^-$ cells after stimulation with *Der p*. interestingly, we observed a decreased MFI for FOXP3 in AC-patients after culture than HC in PBMC. In supernatant of PBMC cultures from AC patients, no differences were observed in TGF-β levels after *Der p* stimulation. In contrast, we found increased IL-10 concentration after antigenic stimulation.
Conclusions: Despite we observed higher frequency of $CD4^+$ $CD25^+$ in AC-patients, these cells were $FOXP3^-$, more interesting, the few cells $FOXP3^+$ showed a diminished MFI. The increase in IL-10 but not TGF-β, may be associated with Th2 dominant environment. These

data suggest that allergic conjunctivitis status could be related with a regulatory dysfunction, as has been suggested in asthma and rhinitis.

P0773

Chimeras of Bet v 1 and its homologue Api g 1 show highly varying degrees of lysosomal stability

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Purpose/Objective: We have previously shown that the major birch pollen allergen Bet v 1.0101 and its homologue in celery Api g 1.0101 differed in their ability to polarise the allergen-specific immune response. In order to identify surface regions responsible for this behaviour, we produced four chimeric proteins of Bet v 1.0101 and Api g 1.0101. In each of them, roughly one fourth of the surface area of Api g 1.0101 were replaced by the corresponding residues of Bet v 1.0101. Resistance to lysosomal degradation enhances immunogenicity. Therefore, our aim was to test the lysosomal stability of these chimeric proteins.
Materials and methods: Chimeric proteins of Bet v 1.0101 and Api g 1.0101 were constructed. The surface residues forming the P-loop (Api-Bet-1), the region opposite of the P-loop (Api-Bet-2), the area surrounding the C-terminus (Api-Bet-3), or the C-terminal alpha helix (Api-Bet-4) of Bet v 1.0101 were grafted onto Api g 1.0101. The resulting proteins were expressed in *Escherichia coli* and purified by standard chromatographic methods. Secondary structures were checked by circular dichroism spectroscopy. For the lysosomal degradome assay, Bet v 1.0101, Api g 1.0101 and the chimeras were digested with mircosomal/endo-/lysosomal enzymes. Reactions were stopped by heat denaturation followed by SDS-PAGE analysis and mass spectrometry.
Results: All chimeric proteins adopted secondary structures equivalent to Api g 1.0101. Results for the degradome assay showed that one of the four chimeric proteins (Api-Bet-2) had a remarkably higher lysosomal stability compared to Bet v 1.0101 and Api g 1.0101. A 50% degradation of Bet v 1.0101 and Api g 1.0101 was observed after 12 h while 80% of Api-Bet-2 remained stable even after 96 h. In contrast, almost total degradation was observed for Api-Bet-1, -3 and -4 after 12 h.
Conclusions: Altering the protein structure when constructing allergen chimeras can result in unexpected increase or decrease of the overall protein stability. In our study, which was supported by grants P22559-B11 (CR) and SFB-F4608 (HB) from the Austrian Science Fund, we produced a chimeric protein whose stability was remarkably greater than the starting molecules Bet v 1.0101 and Api g 1.0101. This change may result in a shift of the immune response polarisation as compared to the wildtype allergens.

P0775

CMRF-35-like molecule 1 (CLM-1) regulates IL-4-dependent responses and is required for allergic eosinophilic airway inflammation

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Purpose/Objective: Asthma is a chronic disease of the airways, which is currently on the rise. IL-4 is a hallmark and central cytokine orchestrating multiple Th2 immune responses/diseases including asthma. Thus, defining pathways regulating IL-4-induced responses may have significant therapeutic potential. CMRF-like-molecule-1 (CLM-1) is an immunoreceptor tyrosine-based inhibitory motif-containing receptor, which is predominantly expressed by myeloid cells. Notably, the *in vivo* role of CLM-1 is unknown and whether it regulates IL-4-induced responses is unclear.
Materials and methods: Wild type (WT) and *Clm1*$^{-/-}$ mice were challenged with *Aspergillus fumigatus* (Asp) extract or IL-4. CLM-1 expression was assessed in the lungs (flow cytometry). Broncho alveolar lavage fluid (BALF) and lung draining lymph nodes were assessed for total and differential cell counts. Th2 cytokine/chemokine content and IgE levels were assessed in BALF and serum, respectively. WT and *Clm1*$^{-/-}$ bone marrow (BM) derived macrophages (MΦ) and eosinophils (Eos) were stimulated with IL-4 and assessed for CCL17 and Relm-α secretion.
Results: CLM-1 was differentially expressed by various lung myeloid cells. Following Asp-challenge, CLM-1 was specifically upregulated by eosinophils and alveolar macrophages. Asp-challenged *Clm1*$^{-/-}$ mice displayed decreased BAL cellular infiltration as well as decreased chemokine and IgE production. Surprisingly, Asp-challenged *Clm1*$^{-/-}$ mice displayed elevated levels of IL-4 suggestive of inappropriate IL-4 consumption by IL-4-responsive cells. Consistently, IL-4-challenged *Clm1*$^{-/-}$ mice displayed decreased BAL cellular infiltration as well as decreased chemokine levels. Indeed, IL-4-stimulated *Clm1*$^{-/-}$ BM-MΦ and BM-Eos displayed significantly decreased Relm-a and CCL17 production in comparison with IL-4-activated WT cells. IL-4R levels were indifferent between WT and *Clm1*$^{-/-}$- cells. Finally, human CLM-1 was upregulated in peripheral blood eosinophils and monocytes obtained from allergic rhinitis patients.
Conclusions: These results demonstrate a key role for CLM-1 in the regulation of allergic airway inflammation likely by regulating IL-4 mediated effects. To the best of our knowledge, this is the first line of evidence suggesting a role for CLM-1 as an activation molecule *in vivo*.

P0776

Contribution of sensitization phase to intensity of contact hypersensitivity reaction in rats

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Purpose/Objective: Allergic contact dermatitis is a common skin inflammatory disease in humans, which pathophysiology is studied mostly on animal model referred to as contact hypersensitivity (CHS). For development of this reaction priming of hapten-specific T cells is necessary (sensitization phase), with subsequent activation of hapten-specific effector T cells, mediators of skin inflammation during elicitation phase. Relationship between sensitizing dose and the intensity of CHS expression was shown in humans, however mechanisms underlying such dose-response are virtually unexplored. In this study, the impact of sensitization phase on the intensity of CHS expression was investigated in a rat model of CHS to dinitrochlorobenzene (DNCB) using low (0.4%) and high (4%) sensitization doses at a constant challenge dose (0.13% or 1.3% DNCB) for each of the sensitizing doses.
Materials and methods: Ear thickness, cell infiltration and IFN-γ content in the medium conditioned by organ-cultured ear skin explants were analyzed during elicitation phase. Basic indices of draining lymph node (DLN) activity (cellularity, proliferation), CD4+/CD8+ composition, the production of interferon-γ (IFN-γ) and

interleukin-17 (IL-17), main effector cytokines in CHS, were analyzed during sensitization and elicitation phases.
Results: Sensitization with high DNCB dose resulted in significantly more intensive inflammatory ear skin response (each of the parameters measured) to challenge with either 0.13% or 1.3% DNCB dose, compared to the response of animals sensitized with low DNCB dose and challenge with respective dose. Higher DLN cellularity, proliferation, CD4+ and CD8+ cell number, and *in vitro* hapten stimulation of IFN-γ and IL-17 production following sensitization with 4% versus 0.4% might have contributed to more pronounced CHS expression in these animals. DLN (auricular) of animals sensitized with 4% and elicited with 0.13% or 1.3% expressed higher proliferation as well as inflammatory cytokine production compared to 0.4%/0.13% or 0.4%/1.3% sensitization/elicitation regime, respectively.
Conclusions: Presented data demonstrated contribution of the strength of sensitization to the intensity of CHS response suggesting that it depends greatly on generation of effector (IFN-γ and IL-17) producing cells.

P0777
Cross-reactivity profiling allows classification of allergenic pectate lyases into two distinct families

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Purpose/Objective: The botanically unrelated families of Asteraceae and Cupressaceae are widely distributed over the Northern hemisphere. Especially in the USA, Europe, and East Asia their pollen represents one of the clinically most relevant allergenic sources. However, the major disease eliciting allergens all belong to the same protein family of pectate lyases. To date, recombinant allergenic pectate lyases showing native folds are not available, thus most studies elucidating pectate lyase allergies were performed with allergen extracts.
Materials and methods: In the present study, the five most important natural allergenic pectate lyases from Asteraceae as well as Cupressaceae pollen were characterized. Therefore, the allergens were purified to homogeneity from crude pollen extracts by standard chromatography techniques. Direct and inhibition ELISA were performed to determine patients' serum IgE binding to the different allergens including sera from four distinct geographical areas (Northern America, the Mediterranean Area, Central Europe, and Asia, respectively). Furthermore, cross-reactivity of purified allergens was assessed in a mouse model.
Results: Purified proteins were characterized physicochemically in terms of identity, structural integrity, and proteolytic stability. The optimized methods allowed purification of structurally intact pectate lyases without detectable auto-proteolysis, degradation, or truncation products. In ELISA experiments, the different cohorts included in the study reacted strongly with the sensitizing pectate lyase, endemic in the particular geographic area. For both, human as well as murine sera, a high degree of cross-reactivity was observed within the pectate lyase families of Asteraceae and Cupressaceae, respectively, whereas between the two families the level of cross-reactivity was limited.
Conclusions: These results allow the classification of allergenic pectate lyases according to their botanical origin in two distinct families, which will provide the basis for the selection of suitable candidate molecules for molecule based allergy diagnosis and therapy.

P0778
Curation of the IUIS allergen database based on sequence similarity and protein family classification

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Purpose/Objective: The IUIS Allergen Database (www.allergen.org) is the official site of the unambiguous and systematic nomenclature of allergens. Allergen names consist of an abbreviation of the scientific name of their source, an allergen and an isoallergen number. We aimed to correct existing database entries based on new sequence information.
Materials and methods: The database was manually searched for entries with missing sequence data, biochemical names similar to those of other allergens from the same source, or inconsistent allergen numbers. Allergen sequences were analysed by pairwise and multiple sequence alignments and phylogenetic trees derived from these alignments.
Results: Four types of incorrect allergen designations were identified. (1) Highly similar allergens from the same source with different numbers. This applies to Amb a 1 and Amb a 2 from ragweed (60−70% sequence identity between Amb a 1 and Amb a 2 isoallergens), to Ara h 3 and Ara h 4 from peanut (91% sequence identity) and to the nine *Chironomus thummi thummi* allergens ($\chi^2 = 1-9$) from the globin family, some with sequences identities >50%. (2) Different numbers for homologous allergens from different species from the same taxonomical family. Sec c 1 from rye and Tri a 28 from wheat are dimeric α-amylase inhibitors. Hor v 21 (γ-hordein) from barley has 76% sequence identity to γ-secalin from *Secale strictum*, a homologue of Sec c 20 from rye. (3) Duplicate entries. Hor v 1 and Hor v 15 from barley refer to the same monomeric α-amylase inhibitor BMAI-1. Equ c 4 and Equ c 5, horse dander latherins, were originally identified based on peptide sequences belonging to the same complete sequence. (4) Single entries referring to a group of different proteins. Caseins from cow's milk are collectively termed Bos d 8. However, caseins comprise four different proteins (αS1, αS2, β, and κ) with sequence identities between α and β-caseins below 20% and no homology to κ-caseins.
Conclusions: Based on these Results: , the IUIS Allergen Nomenclature Sub-Committee renamed Amb a 2.01 to Amb a 1.05, Ara h 4.01 to Ara h 3.02, $\chi^2 = 4-8$ to isoallergens of $\chi^2 = 3$, Sec c 1 to Sec c 28, and Hor v 21 to Hor v 20. The entries Hor v 1 and Equ c 5 were deleted. Bos d 8 was split into four entries: Bos d 8.0101 (αS1-casein), Bos d 9.0101 (αS2-casein), Bos d 10.0101 (β-casein), and Bos d 11.0101 (κ-casein).

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P0779

Decreased NK regulatory cells in children with atopic dermatitis

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Purpose/Objective: Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease characterized by distributed eczematous skin lesions. Numerous studies demonstrated increased frequency of allergen-specific Th2 cells producing increased interleukin (IL)-4, IL-5 and IL-13 in the peripheral blood of AD patients. However little is known about the role of natural killer (NK) cells. NK cells are one component of the innate immune system and have the ability to both lyse target cells and it has been showed that human NK cells are able to polarized functionally different subsets. Recent studies showed that NK cells also display potent regulatory function. In this study, NK1 and NK regulatory cytokine profiles, the expression of activatory receptors as well as the cytotoxic activity of NK cells in AD were investigated.
Materials and methods: The study group consists of children with AD ($n = 8$, mean age = 9.2 ± 3) and healthy subjects ($n = 6$, mean age = 8.6 ± 4). The patients were multisensitized to at least three aeroallergens and had high serum total IgE levels. Peripheral blood mononuclear cells (PBMC) were used as effector cells and K562 cell line was used as target cell with an effector: target (10:1) ratio. Cytotoxic activity (by using CFSE-labeled K562 as target cells), expression of $CD16^{bright}CD56^{dim}$ and $CD16^{dim}CD56^{bright}$ NK cell subsets, NK cell activatory receptors and intracellular IL-10 & IFN-γ levels were also determined by flow cytometry. Statistical analyses were performed by Mann−Whitney *U*-test.
Results: In AD patients $CD16^{bright}CD56^{dim}$, $CD16^{dim}CD56^{bright}$ NK cell subsets, percentages of $CD3^{-}$ $CD16^{+}$ $CD56^{+}$ cells and NK cell cytotoxic capacity were significantly decreasedcompared tohealthy subjects ($P = 0.002$, $P = 0.037$ and $P = 0.007$, respectively). In addition, C type lectin activatory receptor NKG2D/CD94 was found diminished in AD patients in comparison to healthy subjects ($P = 0.05$ and $P = 0.012$, respectively). IL-10 secreting regulatory NK cell ratio dramatically decreased in AD patients, whereas IFN-γsecreting of NK1 cells was found to be similar in both groups.
Conclusions: Our results suggested that decreased expression of $CD16^{bright}CD56^{dim}$, $CD16^{dim}CD56^{bright}$ subsets, activatory receptor NKG2D/CD94, cytotoxic activity and IL-10 secretion of NK cells might play a role in the pathogenesis of atopic dermatitis.

P0780

Design of a hypoallergenic Alt a 1 trimer for specific immunotherapy

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Purpose/Objective: *Alternaria alternata* is one of the major elicitors of fungal allergy and plays a major role in the development of severe respiratory forms of allergy. Alt a 1, a protein of approximately 13 kDa has been identified as the major allergen in *Alternaria*. Alt a 1 binds the majority of *Alternaria*-specific IgE and exhibits high allergenic activity in sensitized patients. The objective of this study was the design, construction and characterization of a hypoallergenic Alt a 1 for specific immunotherapy.
Materials and methods: In order to reduce IgE reactivity and allergenic activity of Alt a 1 the cystein residues stabilizing the proteins fold were mutated to serines. To increase the molecules immunogenicity and ability to induce allergen-specific protective IgG antibodies upon immunization, a recombinant trimeric form of the mutant was expressed in *Escherichia coli*.
Results: The monomeric Alt a 1 serine mutant had completely lost IgE reactivity and a recombinant Alt a 1 trimeric form was expressed in *E. coli* and purified..
Conclusions: The recombinant hypoallergens may be used for specific immunotherapy of allergy to *Alternaria*.

P0781

Design of hypoallergenic derivatives of the major cat allergen Fel d 1 by rational reassembly as a mosaic protein

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Purpose/Objective: Due to the perennial exposure to cats and due to the high association with severe asthma manifestations the major cat allergen Fel d 1 is regarded as one of the most important respiratory allergens. The objective of this study was the rational design of recombinant Fel d 1 derivatives with reduced IgE reactivity and preserved T cell epitopes that are suitable for vaccination and tolerance induction.
Materials and methods: Seven recombinant mosaic proteins were generated by reassembly of non-IgE-reactive peptides of Fel d 1 which contained the sequence elements for induction of allergen specific blocking IgG antibodies and those containing important T cell epitopes. Recombinant mosaic proteins were expressed in *E. coli* using codon-optimized synthetic genes. They were compared with recombinant Fel d 1 regarding their structural fold by circular dichroism, IgE-binding capacity, ability to activate allergic patients' basophils and ability to induce allergen-specific blocking IgG antibodies upon immunization.
Results: Although each of the mosaic proteins had lost the alpha helical fold typical for Fel d 1, a strong reduction in IgE reactivity as well as allergenic activity in basophil activation assays was only obtained for three constructs, two reassembled fragments (Fel d 1 MB, Fel d 1 MC) and a fusion of the latter two in which the cysteines of Fel d 1 MC were replaced by serines. Immunisation of rabbits with the hypoallergens induced high levels of IgG antibodies that inhibited IgE reactivity of cat allergic patients to Fel d 1 in a comparable manner as IgG antibodies induced with the wildtype allergen.
Conclusions: In conclusion, we report the development of hypoallergenic reassembled Fel d 1 proteins suitable for vaccination and tolerance induction in cat allergic patients.

P0782

Development and characterization of recombinant olive pollen allergens, rOle e 5, 6 and 8 for diagnosis and therapy of olive pollen allergy

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Purpose/Objective: Olive pollen is one of the most important causes of allergy in Mediterranean countries and certain parts of America. Ole e 1 an approximately 20 kDa protein is the major allergen in olive pollen which is recognized by more than 90% of olive pollen allergic patients. The aim of our work was to investigate the prevalence of IgE recognition of three olive pollen, Ole e 5 a superoxide dismutase, Ole e 6, a 10 kDa protein with yet unknown biological function and Ole e 8, a calcium-binding protein.
Materials and methods: Thirty sera from clinically well characterized patients with olive pollen allergy from Spain were tested for IgE reactivity to olive pollen extract and purified Ole e 1 by immunoCAP measurements. Recombinant olive pollen allergens, rOle e 5, rOle e 6 and rOle e 8 were expressed as C-terminally hexahistidine-tagged proteins in *Escherichia coli* and purified using Ni-NTA agarose. The structure of rOle e 5, rOle e 6 and rOle e 8 were studied by UV-circular dichroism. IgE reactivity with sera from olive pollen allergic patients was tested by ELISA.
Results: Circular dichroism showed that the recombinant allergens were folded proteins with a predominant beta sheet structure in the case of rOle e 5 and rOle e 8 and alpha helical structure in the case of Ole e 6. Despite symptoms of olive pollen allergy, only twenty six of the patients were positive when tested for IgE reactivity with total olive pollen extract. Twenty three showed IgE reactivity to the major olive pollen allergen Olee 1 (78%), 57% of exhibited IgE reactivity with rOle e 5.20% and 27% of patients reacted with Ole e 6 and Ole e 8 respectively. Interestingly, all four patients negative for olive pollen extract could be detected with a combination ofrOle e 5, rOle e 6 and rOle e 8.
Conclusions: We have produced three recombinant olive pollen allergens rOle e 5, rOle e 6 and rOle e 8 which are useful for component-based testing of olive pollen allergy and eventually for allergen-specific immunotherapy of olive pollen allergy.

P0783

Development of A Rhinovirus-Induced House Dust Mite Asthma Exacerbation Mouse Model

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Purpose/Objective: Rhinovirus (RV) causes the majority of virus-induced asthma exacerbations. The purpose of the present study was to establish an asthma exacerbation mouse model by combing a mouse model of allergic asthma using a clinically relevant allergen house dust mite (HDM) with intra-tracheal inoculation of RV.
Materials and methods: Mice were sensitized and challenged with HDM or saline, and infected with RV-1B, a minor group RV which binds to mouse airway epithelial cells or UV-inactivated RV. Bronchoalveolar lavage fluid and lung homogenates were examined for inflammatory cells infiltration, and Th2 cytokine protein and mRNA expressions. Airway hyperresponsiveness was measured using direct airway resistance analysis.
Results: Compared to HDM/UV-inactivated RV-treated mice, HDM/RV-treated mice developed significant increases in airway infiltration of neutrophils, eosinophils and lymphocytes. The HDM/RV-treated group also produced higher protein levels of IL-4, IL-5, IL-13. Besides, the HDM/RV-treated group generated higher mRNA levels of eotaxin-1 and MUC5AC. However, although we found significant difference in airway hyperresponsiveness to methacholine challenge between saline/RV-treated group and saline/UV-inactivated RV-treated group, the difference between HDM/UV-inactivated RV-treated group and HDM/RV-treated group did not reach significant level.
Conclusions: Taken together, we have developed a model of RV-1B-induced exacerbation of the Th2 responses in HDM-mediated allergic asthma. Additional works are required to illustrate enhanced airway hyperresponsivness in HDM/RV-treated mice. This new asthma exacerbation model will be useful to study asthma immunology and for novel drug discovery and development.

P0784

Different TLR agonists exert diverse effects on immune cells from allergic individuals

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Purpose/Objective: Toll-like receptor (TLR) ligands have been considered as promising adjuvants in vaccines for allergen-specific immunotherapy. We compared different TLR-ligands, namely Pam3CSK4 and FSL-1, targeting TLR2, lipopolysaccharide (LPS) and monophosphoryl lipid (MPL) A, targeting TLR 4 and flagellin, targeting TLR5, regarding their immunostimulatory effects on cells from allergic donors.
Materials and methods: Peripheral blood mononuclear cells (PBMC) were stimulated with TLR-ligands in the presence or absence of autologous plasma and the levels of pro- and anti-inflammatory cytokines were assessed in supernatants. Monocyte-derived dendritic cells (mdDC) were tested for upregulation of CD40, CD80, CD83, CD86, CD58, CCR7 and PD-L1 in response to TLR ligands. Their functional activity was tested in mixed leukocyte reactions (MLR). Moreover, their expression of different members of the IL-12 family was assessed by real-time PCR. $CD4^+$ $CD45RA^+$ T cells were incubated with autologous TLR-activated mdDC to study their polarizing potential to induce Th1- or Th2-like responses. Finally, the influence of TLR ligands on allergen-uptake by monocytes was analysed using fluorescently-labelled Bet v 1, the major birch pollen allergen.
Results: All TLR ligands induced TNF-α and IL-10 synthesis in PBMC from allergic patients whereas only TLR4 ligands induced IFN-γ production. The presence of autologous plasma elevated all cytokine levels. All TLR ligands induced the functional maturation of mdDC. However, different expression patterns of surface molecules on mdDC in response to different TLR ligands were found. LPS-matured mdDC primed Th1-like cells. In contrast, MPL-A, Pam3CSK4 and FSL-1 promoted Th0-like responses, whereas flagellin-matured mdDC induced the differentiation of Th2-like cells. All TLR ligands except flagellin enhanced the uptake of allergen by APC.
Conclusions: Activation of TLR4, TLR5 and the heterodimers TLR1/2 and TLR2/6 has different effects on functional maturation and allergen-uptake of antigen-presenting cells from allergic patients, thereby resulting in different T cell-polarizing capacities. Our data indicate that not all TLR-ligands are equally well suited as adjuvants in allergy vaccines.

P0785

Directed *in vitro* evolution as a tool for the identification of conformational IgE epitopes of the major birch pollen allergen Bet v 1

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Purpose/Objective: Only little is known about the IgE-binding epitopes of the major birch pollen allergen Bet v 1, which have been shown to depend on the native structure of the allergen. In order to identify conformational epitopes, we performed directed *in vitro* evolution of non-IgE-binding structural homologues to proteins carrying single Bet v 1 epitopes. To this end, we chose (S)-norcoclaurine synthase (NCS) from *Thalictrum flavum* (meadow rue), a structural homologue of Bet v 1 with only 25% sequence identity as a template subjected to a combination of random mutagenesis and phage display.
Materials and methods: NCS was expressed in *Escherichia coli* and purified by chromatographic methods. Secondary structure was verified by circular dichroism spectroscopy. IgE binding to birch pollen allergic patients' sera was tested by IgE ELISA. Random mutagenesis of NCS was performed by PCR using mutagenic nucleotide analogues. Phage display libraries in the filamentous phage M13 were created by inserting PCR products into the phagemid pTP127. Bet v 1-specific antibodies were purified from a pool of Bet v 1-sensitised patients' sera by affinity chromatography using immobilised Bet v 1. Several panning rounds were performed to select phages with binding activity to Bet v 1-specific IgE. IgE binding activities of enriched phages and of bacterial colonies representing single clones were analysed by transfer to nitrocellulose and immunostaining.
Results: Purified recombinant NCS revealed a secondary structure with high similarity to that of Bet v 1, but no IgE binding in an ELISA using individual sera from 35 Bet v 1-sensitised birch pollen allergic patients. Phage libraries with diversities of 10^5-10^6 different clones were created. Sequencing of 27 randomly picked clones from the unselected libraries showed average mutation rates of 16/161 amino acids (range 8–24). IgE immunoblotting of phages enriched by two rounds of biopanning showed an increase of the amount of bound IgE, whereas the unselected library lacked IgE binding ability. Screening of single clones after the first panning round yielded 4 of 20 clones which expressed IgE binding mutant proteins.
Conclusions: *In vitro* evolution of NCS by random modifications of surface residues is a suitable tool for defining IgE binding residues on Bet v 1.

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P0786

Dry-roasting enhances peanut immunogenicity and promotes Th2-biased responses in a murine model of peanut allergy

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Purpose/Objective: Peanut allergy is a significant public health concern in many Western developed countries, but its aetiology remains unclear. Thermal processing (including roasting) has been implicated epidemiologically and serologically in peanut allergenicity, but no direct *in vivo* evidence for this putative link has so far been identified. Oxidation has been linked to altered immunogenicity, and our group has previously demonstrated Th2 immunomodulatory activity of oxidation-derived DAMPs such as reactive carbonyl species (RCS). Here we have studied the biochemical properties of DR peanut allergens and have determined their immunogenic and allergenic profiles in a murine model of food allergy.
Materials and methods: Total peanut protein extracts and purified major allergens Ara h 1 and 2/6 were prepared from commercial or in-house dry-roasted (DR) peanuts. To compare the allergenicity of DR with raw peanut allergens, BALB/c mice were s.c. primed with non-crosslinked fractions and subsequently challenged via the intra-gastric route with total peanut extract. Balb/c mice were also directly sensitized and challenged intra-gastrically with total peanut extract.
Results: In line with previous data, DR peanut proteins showed significant increases in RCS content, a hallmark of oxidative stress, and a high level of protein aggregation. Mice immunized s.c. with DR antigens demonstrated a stronger adaptive immune response than those immunized with raw, as revealed by allergen specific Th2-biased T and B cell responses, including a greater induction of functional IgE production. Only mice systemically sensitized by DR-derived allergens responded robustly with Th2 cytokines such as IL-4 and IL-5 in their mesenteric lymph nodes upon gastric challenge. The enhanced allergenic profile of DR peanuts was also confirmed in a direct GI sensitization and challenge model, resulting in enhanced Th2 and IgE responses.
Conclusions: Together these data provide the first direct link between roasting and enhanced peanut allergenicity in a murine model, lending support to epidemiological and serological findings in humans. The enhanced overall immunogenicity of DR peanut cannot be explained by crosslinking alone since this effect was observed even when such species were depleted. Given our preliminary data, the modulated immunogenic profile may be explained in part by the presence oxidation products (including RCS) in DR peanut.

P0787

Ectopic activation of the JAK/STAT pathway leads to loss of barrier function in Drosophila melanogaster airways

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Purpose/Objective: *Asthma bronchiale* is a chronic inflammatory disease of the lungs that is becoming a major health issue in industrialized countries. Asthma pathogenesis is thought to depend on the interaction of genetic and environmental factors but the underlying molecular mechanisms are not well understood. Asthma susceptibility genes like STAT6 are most likely involved in the pathogenesis. For this gene, many functions have been described in asthma related adaptive immune responses. However, the major STAT6 expressing cell type is the bronchial epithelial cell. So far, asthma research has mainly focused on the adaptive immune response. Recently, the epithelium has gained more and more attention. In asthmatics, not only is the epithelium abnormal and has a disturbed barrier function, but also STAT6 expression is enhanced. Therefore it is necessary to analyze the effects of the activation of JAK/STAT signaling on the airway epithelium.
Materials and methods: To address this question we used *Drosophila melanogaster* as a model organism. *Drosophila* has unique advantages for this purpose like the absence of an adaptive immune response and the structure of its airways which consist of epithelial cells only. Furthermore, *Drosophila* has a simplified JAK/STAT signaling system, which is composed of only one representative protein at each position of the cascade.
Results: Here we show that the JAK/STAT pathway can be activated in the airways by hypoxia, a condition important in asthma pathogenesis. Larvae with constitutive JAK/STAT activation do not survive their developmental L2 stadium. A conditional activation after the L2 stadium induces phenotypical changes in the tracheae: liquid filled

branches und melanization, an immune reaction of *Drosophila*. Microarray analyses of these tracheae suggest an involvement of WNT and TGF-β pathways in the induction of this phenotype.
Conclusions: Our data indicate a crucial role of JAK/STAT signaling in the barrier function of the Drosophila airway epithelium. This function is likely to be conserved. Therefore, it is possible that the asthma susceptibility gene STAT6 is important to maintain the barrier function of the human airway epithelium (supported by DFG, SFB/TR22, project A07).

P0788
Effect of boiling and autoclaving on allergenicity of Anisakis simplex

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Purpose/Objective: *Anisakis simplex* is a fish parasite with a worldwide distribution. Humans are accidental hosts. The ingestion of parasitized fish with live larvae L3 can result in the development of anisakiasis and sometimes can induce IgE-mediated reactions. Some authors claim that infection is the only mechanism for allergic disease. However, others consider that allergic episodes can be elicited by infection or exposure to allergen remaining in food with dead larvae. It is believed that is due to heat stable and pepsin resistant allergens for example Ani s 4. The aim of this work was to detect the presence of biologically active heat resistant *A. simplex* allergens after high temperature and pressure treatment (autoclaving) of parasitized fish muscle.
Materials and methods: Fish fillets from hake (*Merluccius merluccius*) and tuna (*Thunnus thynnus*) were artificially parasitized with *A. simplex* and subjected to boiling (10 min) followed by autoclaving during 40 min at 121°C. Fish muscle extracts (FME) were obtained by grinding followed by sonication and centrifugation. *A. simplex* larvae were treated in the same conditions to obtain an L3 extract (ASE). *A. simplex* heat resistant antigens and allergens were detected and quantified in FME and ASE by immunoblot using specific rabbit antisera and/or sensitised patients' sera pool. Autoclaving buffer of the larvae (AB) was also analysed. The FME was tested in a flow cytometry basophil activation test on *A. simplex* sensitized patients. A non parasitized fish muscle extract was used as a negative control.
Results: We could detect several antigens between 6 and 16 kDa and above 36 kDa in the AB but not in the ASE. When revealing with patients' sera pool we detected several allergens between 6 and 16 kDa.

In the FME we mainly detected an allergen around 20 kDa and an antigen around 16 kDa. This extract activated the basophils of sensitized patients in a dose-dependent way.
Conclusions: The presence of heat resistant antigens and allergens from *A. simplex* in artificially parasitized autoclaved fish muscle and autoclaving buffer was demonstrated.

P0790
Efficacious peptide-based immunotherapy in a model of allergic airways inflammation occurs independently of IL-10

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Purpose/Objective: Immunotherapy using short allergen-derived peptides holds promise for the treatment of allergic asthma, and also offers potential as a preventative approach. However, greater understanding of the immunological mechanisms involved in peptide-based immunotherapy (PIT) is required to maximise translational opportunities.
Materials and methods: We have developed a murine model of allergic airway inflammation driven by adoptively transferred Th2 polarised (hence antigen-experienced) ovalbumin (OVA)-reactive $CD4^+$ T cells (OT-II cells). Robust features of allergic airway inflammation are induced in this model following OVA airway challenge, and the Th2 cells are congenically marked, enabling tracking. PIT was applied to this model using the immunodominant OVA T cell epitope pOVA 323–339 given intravenously prior to allergen challenge, and the effects of PIT on disease parameters and immunological readouts were then assessed.
Results: PIT significantly reduced the severity of AAI in this model. Four days after PIT, immediately prior to OVA challenge, PIT treated mice had substantially increased numbers of OT-II cells in lymphoid organs, compared to controls. This is in marked contrast to the early deletional effects of PIT we have previously seen using antigen-naïve OT-II cells. Th2 OT-II cells from PIT treated mice were also unable to induce disease upon direct transfer into the lungs and subsequent OVA airway challenge. Importantly, although Th2 OT-II cells from PIT treated, but not control, mice produced substantial quantities of IL-10 in response to OVA challenge *in vitro*, PIT remained efficacious even in conditions of IL-10R blockade. Furthermore, the therapeutic effects of PIT were evident even in the context of increased severity of allergic airways inflammation induced by IL-10R blockade.
Conclusions: PIT is effective at combating the pathogenic effects of antigen-experienced Th2 cells in a model of allergic airway inflammation. Although PIT induces substantial IL-10 production from Th2 OT-II cells in this model, the therapeutic effects of PIT were not dependent on IL-10.

P0793
Epicutaneous sensitization induces a Der p 2-specific, TH2-biased antibody response independent of TLR4 expression

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Purpose/Objective: The major house dust mite allergen Der p 2 is a structural and functional homologue of MD2 (TLR4-CD14-MD2 complex). An asthma model in TLR4-deficient mice has suggested that the allergic immune response against Der p 2 is dependent on TLR4 signaling. We investigated whether similar mechanisms are important for mediating Der p 2-induced allergy of the skin.
Materials and methods: C57BL/6 WT and TLR4-deficient mice were epicutaneously sensitized 4 times in 3 week intervals with recombinant Der p 2 in combination with or without LPS. Immune response was monitored for specific antibodies by ELISA. Skin sections were analyzed for histological changes and immune cell infiltration.
Results: Co-application of low and high LPS doses with Der p 2 induced similar levels of allergen-specific IgG1 and IgE antibodies in C57BL/6 WT and TLR4-deficient mice. In skin sections, increased

infiltration of mast cells could be determined in both mouse strains, already 6 h after the final challenge. Moreover, we observed high allergenic potential of Der p 2 alone, based on high antibody titers and immune cell infiltration.
Conclusions: Our results suggest that epicutaneous sensitization to Der p 2 does not depend only on activation of TLR-4 signalling but also on other, yet undefined mechanisms.

P0794
Epigenetic effects in mice following ozone exposure

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Purpose/Objective: Ozone exposure can be inducing and inciting factor for asthma. Epigenetics is the study of inherited changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence. The epigenetic effect to ozone exposure needs to be determined. The aim of this study was to investigate the effects of ozone exposure on epigenetic change, airway hyperresponsiveness, and airway inflammation in a murine model.
Materials and methods: BALB/c mice were exposed to 2 ppm ozone for 3 h, 3 days, 7 days, 14 days, and 21 days. Enhanced pause (Penh) to methacholine was measured. Cell differentials in bronchoalveolar lavage (BAL) fluid were done. Epigenetic enzyme such as DNMT1, DNMT3A, MECP2, HDAC3, and MBD1−4 were quantified in the lung tissue homogenate using real time PCR.
Results: The ozone exposure group for for 3 h, 3 days, 7 days, 14 days, and 21 days. demonstrated increased Penh at methacholine concentration of 12.5, 25, 50 mg/ml, with shift in the dose-response curve to the left, compared with that of filtered air group. Neutrophilsand lymphocytes in BAL fluid were increased in ozone exposed group compared with those in filtered air group. After ozone 2ppm exposure DNMT1, MECP2 increased in 21 days and DNMT3a and HDAC3 increased in3 h, 3 days, and 21 days, and MBD1−4 variably increased by ozone exposure time.
Conclusions: These findings demonstrate that ozone exposure can change enzymes related to epigenetics.

P0795
Evidence for T cell-independent boost of allergen-specific secondary IgE responses by B cell epitopes

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Purpose/Objective: IgE-mediated allergy, a hypersensitivity disease affecting more than 25% of population, is characterized by the induction of an allergen-specific Th2 response and the production of allergen-specific IgE antibodies. Clinical studies showed that systemic allergen-specific production in allergic patients is strongly boosted by respiratory allergen contact and that patients become more sensitive to allergen contact. Despite the key role of IgE antibodies for the pathology of allergic disease, the mechanisms underlying allergen-specific secondary antibody responses are still not completely understood. The aim of this study was to investigate what allergen epitopes (B cell, T cell epitopes) are needed for the boosting of allergen-specific secondary IgE responses.
Materials and methods: Based on the hapten-carrier model developed by Benacerraf, a 31 amino acid peptide derived from the major grass pollen allergen Phl p 1, which was devoid of T cell epitopes recognized by BALB/c mice, was coupled to unrelated carrier molecules. Immunization of BALB/c mice with the peptide/carrier allowed the induction of peptides-specific IgE antibody responses. Sensitized mice were then boosted with peptide constructs devoid of carrier-specific T cell epitopes and for control purposes with the original immunogens, the original carrier molecules without peptide or PBS. Peptide-specific IgE responses were determined by ELISA and by using rat basophil degranulation assays.
Results: Interestingly, we found that peptide-constructs devoid of the original carrier molecules and thus devoid of T cell epitopes could induce increases of secondary peptide-specific IgE responses. Application of the T cell epitope-containing carriers alone had no influence on ongoing IgE responses.
Conclusions: Our results suggest that B cell targeting approaches may be needed for the treatment of established allergy.

P0797
Extracellular heat shock protein 70 inhibits impairment of airway neutrophils responses in induced allergic airway inflammation

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Purpose/Objective: Immune responses of airway neutrophils are thought to be impaired in asthma. Stress-associated chaperon heat shock protein 70 (Hsp70) is known to be potent to alter neutrophil activation in response to bacterial infection. Here we analyze the effect of extracellular Hsp70 application on neutrophil infiltration and activity upon induced allergic airway inflammation (IAAI).
Materials and methods: IAAI in BALB/c mice was induced by intraperitoneal ovalbumin (OVA)/Alum injection and subsequent OVA aerosol challenges. A group of mice received intra-pharyngeal injection of murine Hsp70 at 24 h after the last allergen challenge. Total and differential number of bronchoalveolar lavage (BAL) cells and percentage of neutrophils in bone marrow was analyzed at 48 h after the last allergen challenge. Potential of bone marrow neutrophils from Hsp70 treated mice to generate reactive oxygen species (ROS) in response to phorbol-meristil acetate was investigated.
Results: Hsp70 application decreased total BAL cell number and eosinophil percentage, however maintained neutrophil infiltration at 48 h after the last allergen challenge. The percentage of neutrophils in bone marrow of mice that received Hsp70 was comparable to that exhibited allergic inflammation. Simultaneously treatment with Hsp70 restored the ability of bone marrow neutrophils to generate ROS, which was detected to be suppressed in mice with IAAI.
Conclusions: Thus protective mechanism of extracellular Hsp70 in IAAI could be related to inhibition of airway neutrophil responses impairment.

P0798

Functional characterization of allergen-specific T cell responses in a humanised mouse model using a human mugwort-specific T-cell receptor and HLA-DR1

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Purpose/Objective: Chicken Ovalbumin (Ova) is a frequently used model allergen in allergy and asthma research and most *in vivo* experiments are performed in T cell receptor (TCR) transgenic (tg) mice expressing a murine TCR specific for Ova in the context of a murine restriction element ($I\text{-}A^d$). We here aimed to generate double tg mice expressing a human TCR specific for the immuno-dominant epitope of the major mugwort (*Artemisia vulgaris)* pollen allergen Art v 1 in the context of the human restriction element HLA-DR1.

Materials and methods: To obtain high expression levels the allergen-specific human TCR variable sequences were chimerized with murine TCR constant sequences. Resulting transgenes were cloned into the pT_{cass} vector system and thus put under the transcriptional control of the natural TCR alpha and beta promotor/enhancer elements. Allergen-specific TCR tg founder mice were cross-bread with HLA-$DR1^+$ B10.M-$DR1^{dlAb1\text{-}Ea}$ mice.

Results: Immunophenotyping of double tg TCR/HLA-DR1 mice revealed clear-cut expression of the Art v 1-specific TRBV18 chain on peripheral blood $CD3^+$ T cells and HLA-DR1 expression on $CD14^+$ monocytes and $B220^+$ B cells. *In vitro,* splenocytes from TCR/HLA-DR1 double tg mice but not of HLA-DR1 single tg mice or wt mice specifically proliferated upon incubation with the human-relevant immuno-dominant Art v 1_{25-36} peptide or whole Art v 1 protein. No proliferation was observed upon incubation with control peptides or proteins. Allergen-specific cellular proliferation is accompanied by the production of a balanced cytokine milieu including IFN-gamma, IL-2, IL-4, IL-6, IL-13 and IL-17. The effect on T-cell phenotype, specific antibody production and lung function after *in vivo* challenge with specific allergen via the airways will be described and discussed.

Conclusions: A humanised allergy model, in which all components of the allergen-specific synapse are well-defined enables to analyze the relevant T-cell dependent pathways by which allergic diseases can be influenced *in vivo* and will provide important insights into the pathophysiology of allergic diseases and their possible cure in the future.

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P0799

Granulocyte/macrophage colony-stimulating factor-dependent CD11b lung dendritic cells are required for induction of T helper 2 immunity to inhaled dust mite allergen

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Purpose/Objective: Although it is well established that dendritic cells (DCs) are essential for allergic immune responses in several mouse models of asthma but the specific role of different lung DC subsets in initiating Th2 immunity remains poorly understood. The purpose of this study was to investigate the contribution of tissue resident dendritic cells in inducing Th2 immune response to the tropical allergen *Blomia tropicalis*.

Materials and methods: C57BL/6 mice were intra-nasally sensitized with Blomia tropicalis extract (Blo t) and subsequently challenged via the same route. Bronchoalveolar lavage (BAL) eosinophils and neutrophils were determined by expression of Singlec-F and Ly6-G respectively. Mucus secretion was detected with periodic acid Schiff stain (PAS). T cell cytokines were determined by culture of draining lymph node cells with 20 mg/ml Blo t extract for 5 days and assay of culture supernatants by ELISA (R&D systems).

Results: Intra-nasal administration of Blo t without adjuvant induced a robust allergic response characterized by high eosinophil (60%) and low neutrophil (10%) infiltration. Hematoxylin and eosin staining identified substantial mononuclear cell infiltration of the lungs and PAS staining demonstrated mucus hyper-secretion and goblet cell hyperplasia. Draining lymph node cells cultured with Blo t extract secreted large amounts of IL-4 (200 pg/ml), IL-5 (2000 pg/ml), IL-13 (4000 pg/ml) and very little IFN-g (<100 pg/ml). Blo t extract was able to enhance the uptake (10−20% by day 3) and presentation of ovalbumin (OVA) by CD11b lung DCs, leading to OVA-specific T cell proliferation and Th2 differentiation in lung draining lymph nodes. When CD11b lung DCs were depleted by crossing CD11c-Cre and IRF4-flox mice, the Th2 immune response to inhaled Blo t allergens was attenuated and allergic airway inflammation decreased. Administration of anti-granulocyte/macrophage colony-stimulating factor (GM-CSF)-neutralizing Abs during the sensitization stage markedly reduced Blo t-elicited allergic responses and was associated with reduced antigen presentation capacity of CD11b lung DCs.

Conclusions: Taken together these data suggest that GM-CSF-dependent CD11b DCs play a critical role in the initiation of Th2 responses to Blo t allergens.

P0800

Heroin and histamine

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Purpose/Objective: In 2011 more than 200 narcotic drug-related deaths have been verified in Austria. To support the hypothesis that anaphylaxis is involved in long-term drug addiction as a possible cause of death we investigated useful markers measured in baseline bloods of long-term heroin addicts.

Materials and methods: Quantitative laboratory tests were performed to determine the concentrations of Histamine, Diamine oxidase (DAO), Tryptase and Lipoprotein-associated phospholipase A_2 ($LpPLA_2$) in drug addicts ($n = 72$) and healthy controls ($n = 103$).

Blood samples of drug addicts were taken before and 3h after substitution (Substitol®retard).

To verify the hypothesis, we documented self-reported anaphylactic reactions shown during drug addiction plus the expression of anaphylactic symptoms after substitution within 180 min.

Results: We found significantly elevated Histamine (0.51 ± 0.33 ng/ml versus 0.18 ± 0.15 ng/ml; $P < 0.001$), Tryptase (5.53 ± 3.93 versus 3.96 ± 1.99 μg/l; $P < 0.001$) and $LpPLA_2$ (399.71 ± 118.04 ng/ml versus 333.28 ± 85.95 ng/ml; $P < 0.001$) levels in heroin addicts compared with healthy controls, while DAO (14.00 ± 7.73 versus 11.02 ± 4.91 U/ml; $P < 0.001$) was significantly decreased.

Anaphylactic reactions during long-term drug addiction were reported in 69 of 72 cases. Skin involvement (urticaria, flushing, and pruritus) is reported in 60%, respiratory tract involvement (dyspnoea and angioedema) in up to 50%.

Prescription of substitution has caused side effects merely in 13 of 72 cases. Unspecific symptoms like tiredness, feeling of warm, headache and pruritus have been documented.

Conclusions: These results show 3-fold increased Histamine concentrations ($P < 0.001$), elevated Tryptase ($P < 0.001$), and $LpPLA_2$ ($P < 0.001$), concentrations in baseline bloods of heroin addicts compared with healthy controls, DAO ($P < 0.001$) was reduced. It indicates that long-term heroin consumption influences the release of mast-cell mediators. Hence, anaphylactic reactions were described in long-term drug addicts, containing the skin and the respiratory tract as the main organs.

These observations suggest that raised histamine may induce arrhythmias and ischaemic changes which finally may be a cause of heroin-induced fatalities. This study however emphasizes, that heroin might be an important trigger of anaphylaxis.

P0801
Heterogeneity of allergen-specific Th2 cells in Type I allergy

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Purpose/Objective: The important roles of allergen-specific Th1, Th2 and Treg cells in IgE-mediated allergy have been well defined in the past. However, the involvement of Th17 cells in Type I allergy is not clear yet. Therefore, we investigated the existence of allergen-specific Th17 cells.

Materials and methods: Supernatants of 40 allergen-reactive $CD4^+$ clones expanded from peripheral blood of allergic patients were assessed for cytokine production after specific stimulation using the Luminex System. The expression of lineage-specific transcription factors of the clones was analyzed by qPCR and compared to *in vitro* differentiated Th1, Th2 or Th17 cells. Intracellular cytokine staining of allergen-stimulated peripheral blood mononuclear cells and clones was done by flow cytometry.

Results: Twenty two of 40 allergen-specific clones (55%) produced IL-4 and negligible amounts of IFN-g and were thus considered as Th2 cells. In addition to IL-4, these clones produced TNF-α, IL-10 and/or IFN-γ showing high heterogeneity within the Th2 lineage. Nine of 40 clones (23%) produced IL-17 upon stimulation with allergen. Interestingly, all IL-17-producing clones concomitantly synthesized substantial amounts of IL-4. Analysis of lineage-specific transcription factors showed that both, $IL\text{-}4^+IL\text{-}17^-$ and $IL\text{-}4^+IL\text{-}17^+$, clones display expression profiles characteristic for Th2 cells. *Ex vivo*, the existence of allergen-specific $IL\text{-}4^+IL\text{-}17^+$ T cells in peripheral blood from allergic individuals was confirmed by intracellular cytokine staining of allergen-stimulated proliferating T cells.

Conclusions: We found no evidence for allergen-specific Th17 cells causing IgE-mediated allergy. However, we identified a subset of allergen-specific $IL\text{-}4^+IL\text{-}17^+$ Th2 cells and observed high heterogeneity of allergen-specific Th2 clones.

P0802
Hypoallergenic Der p 1/Der p 2 combination vaccines for immunotherapy of house dust mite allergy

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Purpose/Objective: More than 50% of allergic patients suffer from house-dust mite (HDM) allergy. Group 1 and 2 allergens are the major HDM allergens.

Production and preclinical characterization of a recombinant hypoallergenic combination vaccine for specific immunotherapy (SIT) of HDM allergy.

Materials and methods: Synthetic genes coding for two hybrid proteins consisting of reassembled Der p 1 and Der p 2 fragments with (rDer p 2/1C) and without (rDer p 2/1S) cysteines were expressed in *Escherichia coli* and purified to homogeneity by affinity chromatography. Protein fold was determined by circular dichroism analysis, allergenic activity by testing IgE reactivity as well as basophil activation assays and presence of T cell epitopes by lymphoproliferation in allergic patients. Mice and rabbits were immunized to study the molecules ability to induce an allergic response and if they induce allergen-specific IgG capable of inhibiting allergic patients' IgE binding to the allergens, respectively.

Results: rDer p 2/1C and rDer p 2/1S were expressed in large amounts in *E. coli* as soluble and folded proteins. Due to lack of disulfide bonds, rDer p 2/1S did not form aggregates and was obtained as monomeric protein, whereas rDer p 2/1C formed aggreates. Both hypoallergens lacked relevant IgE reactivity and had reduced ability to induce allergic inflammation and allergic responses, but induced similar T cell proliferation as the wildtype allergens. Immunization with the hypoallergens (rDer p 2/1S > rDer p 2/1C) induced IgG antibodies in rabbits that inhibited IgE reactivity of HDM-allergic patients to Der p 1 and Der p 2.

Conclusions: The preclinical characterization indicates that in particular rDer p 2/1S may be used as a safe hypoallergenic molecule for tolerance as well as vaccination approaches to treat HDM allergy.

P0803
Identification and characterization of pulmonary stem cells in the pathogenic mechanism and alleviation of allergic airway inflammation

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Purpose/Objective: Asthma is a very complex and heterogeneous disease that is characterized by airway inflammation and airway hyperresponsiveness (AHR). Recent studies have been focused on characterization and identification of lung stem cells for regenerative therapy in asthma. Based on the theory of alveolar development in the newborn lung, pulmonary stem cells are enriched in the neonatal mice

and becoming the rare population in the well-development adult mice. Therefore, we isolated and explored the biological effect of the potential SSEA-1^+ pulmonary stem cells (PSCs) derived from neonatal mice.

Materials and methods: SSEA-1^+ PSCs derived from neonatal mice were enriched by magnetic beads. The characteristics of SSEA-1^+ PSCs were evaluated using immunofluorescence staining, real-time PCR, and FACS analysis. Further, *in vivo* biological function of SSEA-1^+ PSCs was studied and analyzed in OVA-induced asthmatic mice.

Results: Single-cell suspensions derived from neonatal mouse lung tissue were SSEA-1^+ or Sca-1^+ population. SSEA-1^+ PSCs were highly expressed in the neonatal mice, and then were downregulated in the adult mice. Enriched SSEA-1^+ PSCs have the ability to differentiate into AQP5^+ type I pneumocytes and surfactant protein C-expressed type II pneumocytes. FACS and real-time PCR showed that CCSP transcript and protein level were increased in SSEA-1^+ PSCs compared with SSEA-1^- Sca-1^- pulmonary cells. Adoptive transfer of SSEA-1^+ PSCs reduced AHR and prevented airway damage through decreasing eosinophil infiltration and inhibiting IL-5, eotaxin and TSLP production in OVA-induced asthma mice.

Conclusions: In conclusion, SSEA-1^+ PSCs might have the potential to repair lung damage or to inhibit inflammatory effects in the pathological process of asthma. Therefore, we conclude that the potent immunomodulatory effect of PSCs might be beneficial for treating asthma disease.

P0804

Identification of a major IgE-binding epitope of beta-parvalbumins using scFv antibodies

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Purpose/Objective: Fish allergy is associated with IgE-mediated hypersensitivity reactions to β-parvalbumins, which are small calcium-binding muscle proteins, and represent the major for 95% of fish allergic patients. A high degree of cross-reactivity of parvalbumins from various fish species has been described. We aimed to express and purify single chain variable fragment (scFv) antibodies specific for β-parvalbumins for their detection in fish protein extracts and for the identification of major IgE epitopes.

Materials and methods: The phage clone gco9 specific for β-parvalbumins was isolated from the ETH-2 phage display library by sequential panning against parvalbumins from Atlantic cod, carp and rainbow trout. The soluble scFv-gco9 was expressed in the *E. coli* non-suppressor strain HB2151 and extracted from the periplasm. The purified antibody was obtained by nickel-based affinity chromatography and its specificity was tested in Western blot using fish protein extracts. The binding capacity of scFv-gco9 for the natural parvalbumins Gad m 1, Cyp c 1 and Onc m 1 of Atlantic cod, carp and rainbow trout, respectively, was tested in ELISA. Inhibition ELISAs were performed to investigate the ability of this antibody to block sIgE-binding to β-parvalbumins in sera of fish allergic patients.

Results: The purified scFv-gco9 was able to detect β-parvalbumins of Atlantic cod, carp and rainbow trout as single 12 kDa bands in Western blot using fish protein extracts. The antibody also strongly recognized natural Gad m 1, Cyp c 1 and Onc m 1 in direct ELISA. The binding capacity of scFv-gco9 to nGad m 1 was independent of the presence or absence of Ca^{2+} ions. In contrast, a mouse monoclonal anti-parvalbumin antibody only reacted with the calcium-bound form. In competitive ELISA, the scFv antibody was able to inhibit binding of sIgE from fish allergic patients' sera to all three β-parvalbumins by 80%.

Conclusions: In this work, supported by grant SFB-F4608 from the Austrian Science Fund, we isolated and produced a scFv antibody specific for a calcium-independent major IgE epitope of Atlantic cod β-parvalbumin.

P0805

IgE knock-in mice reveal a key role for IgE in basophil-mediated active systemic anaphylaxis

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Purpose/Objective: IgE production is tightly regulated at the cellular and genetic level and is believed to be central to allergy development. At least two pathways exist that lead to systemic anaphylaxis reactions *in vivo*: IgE-sensitized mast cell and IgG1-sensitized basophil mediated anaphylaxis. Although passive anaphylaxis, by application of allergen and allergen-specific antibodies in mice allows for the determination of the contribution of different immunoglobulin isotypes, the analysis of a dynamic, immunization mediated antibody responses is not currently possible.

Materials and methods: We generated IgE knock-in mice (IgE^{ki}) which express the IgE heavy chain instead of IgG1 in order to analyze the contribution of IgG1 and IgE to active anaphylaxis *in vivo*.

Results: IgE^{ki} mice display increased IgE production both *in vitro* and *in vivo*. In addition a defect of membrane expression of chiemric IgE *in vivo* was observed, which suggests that mIgE expression id fundamentally differenet from mIgG1 expression *in vivo*

The sensitization of IgE^{ki} mice by immunization followed by antigen challenge leads to increased anaphylaxis. Homozygous IgE^{ki} mice, which lack IgG1 due to the knock-in strategy, are most susceptible to active systemic anaphylaxis. Depletion of basophils demonstrates their importance in IgE-mediated anaphylaxis.

Conclusions: Therefore we propose that an enhanced-, antigen-specific, polyclonal, IgE response, as is the case in allergic patients, is the most efficient way to sensitize basophils to cause systemic anaphylaxis *in vivo*.

P0806

IgE reactivity profiling of allergic individuals in the Philippines

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Purpose/Objective: There is only limited information about allergy and relevant allergens in the Philippines. This study aimed to investigate the allergen profile in the Philippines.

Materials and methods: A questionnaire for Allergy Screening from the International Study of Asthma and Allergy in Childhood was used to identify individuals with and without allergic symptoms. Allergen specific-IgE antibodies were determined by ELISA using pollen extracts from several local grasses such as *Cynodon dactylon, Chloris barbata, Imperata cylindrica, Saccharum spontaneum, Sporobulus indicus, Oryza sativa* and *Zea mays*. In addition, chips containing 103 micro-arrayed purified allergen molecules were used to determine the molecular IgE reactivity profiles in random subgroups of 79 symptomatic and 20 asymptomatic subjects. IgE binding studies were performed in purified rOry s 1 from rice and galactose-α1,3-galactose (α-Gal); and IgE inhibition assay with glycosylated nPhl p 4 was done. Nitrocellulose-blotted extracts from local grasses were analyzed for the presence of major grass pollen allergens with specific antibody probes.
Results: Interestingly, we found that not only symptomatic subjects but also asymptomatic individuals exhibited IgE reactivity to pollen extracts of local grasses. A detailed characterization of the IgE reactivity profiles with micro-arrayed allergens revealed an unusual preferential sensitization to mainly glycosylated grass pollen allergens such as nCyn d 1 and nPhl p 4 in both groups whereas the major protein allergens in grass pollen were not recognized. The specificity of the IgE reactivity to carbohydrate epitopes with poor allergenic activity was confirmed by IgE inhibition studies. No sensitization to the allergenic α-Gal was observed. Symptomatic allergic sensitizations were confined to non-glycosylated protein allergens from house dust mites and animals.
Conclusions: Our study reveals that subjects from the Philippines exhibit a high prevalence of asymptomatic IgE sensitization to non-allergenic carbohydrate epitopes in grass pollen whereas symptomatic allergic sensitization seems to be confined to protein allergens from mites and animals. These results have impact on the development of allergen-specific forms of immunotherapy.

P0807
Immature dendritic cells in patients with ill-controlled asthma

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Purpose/Objective: Ill-controlled allergic asthma, a chronic inflammatory disease, has been increasingly observed in both developed and developing countries. Inhalant steroid has been applied to control disease that is transient used to reduce disease severity, however, which could not be used to cure disease completely. Therefore, to approach alternative pathogenic mechanisms involved asthma facilitates to develop new therapeutic targets. Previous studies revealed Th2 cells and other inflammatory cells play a major role in the initiation and pathological development of asthma, therefore, allergenic antigens specific adoptive immune responses play an important role in pathogenic mechanisms of disease. Dendritic cells (DCs) are professional antigen presenting cells that play a crucial role to establish adoptive immune responses to various inflammatory stimuli. The well accepted concept that human blood monocytes can be induced to develop immature DCs after treating GM-CSF and IL-4.
Materials and methods: In our study, MoDCs were used to evaluate whether function of DCs involved pathogenicity of asthma that MoDCs derived from 6 patients with ill-controlled asthma or derived from 10 health donors. All of patients were discontinued to treat systemic or inhalant steroid before blood collection.
Results: MoDCs derived from asthma patients presented lower levels of accessory molecules, including CD40, CD80, CD83, CD86 or MHC class II compared with those molecules derived from MoDCs of healthy donors. Consistently, MoDCs cultured from patients presented potent capability to uptake fluoresce-conjugated model antigen than MoDCs derived from health donors. In addition, results of mix lymphocytes reaction revealed that MoDCs generated from patients have a weak capacity to prime naïve CD4+ T cells proliferation compared with those derived from health donors. These data indicate that patients with ill-controlled asthma have less potency of MoDCs. Interestingly, both MoDCs derived from patients and health donors were activated and maturated after LPS stimulation, however, most of MoDCs cultured from asthma patients still have shown weak potency to prime naïve T cells than those generated from health donors.
Conclusions: These data indicate that less potent and immature DCs in patients with ill-controlled asthma might be play a role in pathogenic mechanism of disease.

P0808
Immune mechanisms mediating suppression of allergic airway inflammation upon *Salmonella* infection

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Purpose/Objective: Prevalence of asthma, a Th2 biased inflammatory airway disease has dramatically increased over the past few decades. Mere reciprocal regulation of Th1 and Th2 phenotypes failed to justify the concomitant rise in allergic and autoimmune disorders characterized by dys-regulated Th1 or Th17 immune responses. Recent studies have highlighted the role of immune-regulatory mechanisms in modulating allergic reactions. A longitudinal study by Pelosi *et al.* (Allergy, 2005) demonstrated an inverse correlation between *Salmonella* infection and susceptibility to asthma, however, the mechanism remains obscure. In this study we attempted to identify the mechanism(s) mediating this suppression.
Materials and methods: Experimental allergic airway inflammation was induced by standard OVA-Alum model. A group of mice were infected with *Salmonella* during the regimen. Inflammatory responses were estimated by cellular infiltration in broncheo-alveolar lavage (BAL). Serum immunoglobulin levels and cytokines from *in vitro* re-stimulated mediastinal lymph node cells were measured by ELISA. Lung pathology was determined by histology and quantitative PCR. Alteration in cellular frequencies was determined by flow cytometry. *In vitro* differentiation and stability of Th2 cells were investigated.
Results: Mice infected with *Salmonella* showed markedly reduced total cellular infiltration and eosinophilia in the BAL. Infected mice showed increased titers of antigen-specific IgG2a in sera, but no significant alteration in IFN-g was detected. Reduced IL-4 secretion was observed which correlated to decreased MUC5AC expression in the lungs. No demonstrable change in the frequencies of $Foxp3^+$ Tregs was observed. Infected mice showed a significant increase in cells expressing $CD11b^+/Gr1^+/Ly6C^{int}$. Using *in vitro* co-cultures we demonstrate that $CD11b^+/Gr1^+/Ly6C^{int}$ cells do not inhibit Th2 differentiation but destabilize Th2 phenotype by down-modulating GATA-3 expression.
Conclusions: We observed amelioration of airway inflammation in *Salmonella* infected mice. This suppression was Treg independent. No substantial shunt towards a Th1 phenotype was detected. We demonstrate expansion of $CD11b^+/Gr1^+/Ly6C^{int}$ cells upon *Salmonella* infection, which exert their suppressive function by influencing the Th2 stability. This could be a potential mechanism of protection from asthma.

P0809

Immunological reactions to the carbohydrate antigen alpha-Gal

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Purpose/Objective: Galα1-3Galβ1-4GlcNAc-R (alpha-Gal) is a carbohydrate expressed by non-primate mammalians. It is the main reason of hyperacute graft rejection in xenotransplantation. Recently, alpha-Gal has been described as an important allergenic structure in red meat allergy. Since the role of carbohydrates in allergy is still controversial, we sought to investigate the relevance of alpha-Gal in meat allergy and to identify proteins carrying the allergenic sugar.

Materials and methods: Twenty patients who had experienced generalized urticaria after consuming meat were included. All patients showed IgE-reactivity to beef or pork in ImmunoCAP. IgG and IgE antibodies specific for alpha-Gal were analysed by ELISA. Protein extracts were produced from beef and pork and used in immunoblots to test IgE-reactivity to individual meat proteins. Additionally, sera from 15 patients receiving a biological heart valve and 8 patients receiving mechanical prostheses obtained preoperatively, 10 days and 3 months postoperatively were analysed.

Results: All meat-allergic patients displayed alpha-Gal-specific IgG and 19/20 patients showed alpha-Gal-specific IgE. In immunoblots, 13/19 alpha-Gal sensitized patients showed IgE specific for a protein of approximately 160 kDa, equivalent to bovine gamma globulin (BGG). The same protein was detected to be glycosylated. In inhibition-ELISA, IgE-reactivity to BGG was completely abolished after pre-incubation with alpha-Gal. The majority of biovalve recipients displayed high levels of alpha-Gal-specific IgG which increased in 12/15 individuals 10 days post surgery. Five of these patients showed increased alpha-Gal-specific IgE levels already 10 days post surgery. None of the patients with mechanical bioprostheses showed a rise in alpha-Gal-specific antibodies.

Conclusions: Our data indicate that IgE-reactivity to alpha-Gal on BGG plays an important role in meat allergy. We currently investigate why the presence of high levels of alpha-Gal-specific IgG antibodies is incapable of blocking IgE-binding to the carbohydrate. The occurrence of alpha-Gal-specific IgE in biovalve recipients post surgery indicates, that sensitization to alpha-Gal can develop although alpha-Gal-specific IgG is present.

P0810

Immunomodulatory and bronchodilatory potential of *Allium cepa* L. and its flavonoid quercetin *in vitro*

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Purpose/Objective: This study was conducted investigating the *in vitro* anti-inflammatory activity and bronchodilator potential of the methanolic extract of the *Allium cepa* L. *(AcE)* and its flavonoid quercetin (Qt) on the production of IL-4, IL-5 and IL-13 by spleen cell; production of nitric oxide on peritoneal macrophages cultures, and their bronchodilator potential on isolated trachea from mice.

Materials and methods: To this end, the allergic process was induced in A/J mice by the administration of *Blomia tropicalis* antigen. The animals were sensitized with 100 μg per animal − s.c. in 4 mg/ml of [AL $(OH)^3$] on days 0 and 7; after the last sensitization the animals received four intranasal challenged (10 μg per animal − i.n) with *Blomia tropicalis* (*BtE*) mite at intervals of one day. Spleen cells isolated from allergic animals were incubated with different concentrations of *AcE* and Qt, stimulated or not with pokeweed mitogen (PWM, 2.5 μg/ml); cultures were then incubated for 2 days and supernatants were collected for cytokine measurement. Peritoneal macrophages were isolated from normal mice and incubated with different concentrations of *AcE* and Qt for 1 h in the presence or not of LPS (concentration) for 24 h. After this period, the supernatants were collected and analyzed for nitrite by Griess reaction. The amount of nitrite in the sample was determined using a sodium nitrite standard curve. Additionally, trachea from non sensitized mice were sectioned into rings, and placed in tanks for isolated organ with Krebs-bicarbonate solution and aerated with a carbogen mixture (95%O_2 and 5% CO2). The rings were contracted with carbachol to evaluate the presence of functional epithelium, and after reaching a plateau of contractile state, the rings were stimulated with bradykinin. Cumulatively increasing concentrations of *AcE* or Qt were added. Concentration response curve was constructed and the data were analyzed.

Results: The production of inflammatory cytokines (IL-4, IL-5 and IL-13) and nitric oxide was significantly reduced by the treatment with different concentrations of *AcE* and Qt when compared with positive control. Also was observed a dilator effect of *AcE* and Qt on carbachol-induced contraction in isolated trachea.

Conclusions: The results obtained in this study suggest that *Allium cepa* L. and Qt have potential as anti-asmatic drugs by both immunomodulatory and bronchodilatory properties. The molecular mechanisms whereby*Allium cepa* L. and Qtact are under exploration by our group.

P0812

Induction of mucosal tolerance with structurally different antigens: studies on underlying mechanisms

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Purpose/Objective: In a mouse model of poly-sensitization, we have shown that intranasal administration of a linear synthetic hybrid, composed of immunodominant T-cell epitopes of the major birch and grass pollen allergens Bet v 1, Phl p 1 and Phl p 5, induced tolerance in mice sensitized with the 3 allergens. Tolerance induction was associated with reduced allergic inflammation and increased production of the regulatory cytokine IL-10 in the lungs. On the other hand, tolerance induction with the whole recombinant (*r*) Bet v 1 led to reduction of airway inflammation within the lung without the induction of IL-10 in the lung. Thus, we want to understand the mechanistic pathways of tolerance induction by these two structurally different antigens by investigation of their uptake and presentation by antigen presenting cells.

Materials and methods: In order to follow up their internalization, hybrid and rBet v 1 were conjugated with the fluorescence dye 5 (6)-Carboxyfluorescein (FAM). For *in vivo* studies, constructs were administered intranasally. After various time points (0, 1, 6, 24 and 48 h) the antigen uptake in the nasal-associated lymphoid tissue (NALT), lung, bronchial lymph nodes, spleen and blood was investigated by flow cytometry. Furthermore, FAM-hybrid and FAM-rBet v 1 were co-cultured with isolated murine mononuclear lung cells *in vitro* in a time dependent manner (0, ½, 1, 6 and 24 h) and cells capturing these antigens were identified and characterized.

Results: *In vivo*, both antigens were taken up by two main populations: CD11c(+) CD11b(+) macrophages and CD11c(+) CD11b(−) dendritic cells in the lung and NALT. Interestingly, more FAM-hybrid than FAM-rBet v 1 molecules were detected within the lung cells. None

of the investigated antigens have been detected in the spleen or in the blood. *In vitro*, macrophages, dendritic cells and additionally B cells were internalising the antigens, and B cells displayed the population with the highest antigen uptake capacity. With respect of the time kinetic these B cells internalised the FAM-hybrid about 6 h earlier than the FAM-rBet v 1.

Conclusions: *In vivo* and *in vitro* kinetic studies demonstrate that there are differences between the antigen uptake of the linear multipeptide (hybrid) and the conformational allergen (rBet v 1). Further studies on internalisation pathways are currently ongoing.

P0813

Inflammatory marker sTREM-1 reflects the clinical stage and respiratory tract obstruction in allergic asthma bronchiale patients and correlates with number of neutrophils

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Purpose/Objective: TREM-1 (Triggering receptor expressed on myeloid cells), is constitutively expressed on the surface of myeloid cells-neutrophils, monocytes and macrophages. The membrane form of TREM-1 can be cleaved from the surface of activated myelocytes and its soluble form (sTREM-1) is released into microenvironment. The highest levels of sTREM-1 have been found in patients with sepsis and other inflammatory diseases caused mainly by extracellular microorganisms as well as inflammatory states of non-infectious origin. The knowledge that asthma bronchiale is an inflammatory disorder has prompted us to investigate the plasma levels of new inflammatory marker sTREM-1 that is released from the surfaces of activated neutrophils and monocytes.

Materials and methods: The plasma levels of sTREM-1 were analysed by a sandwich Elisa test in the cohort of 76 patients with allergic asthma bronchiale and 39 healthy controls. Either Anova or Mann−Whitney *U*-test were used to determine the difference and the statistical significance. The association of sTREM-1 plasma levels with clinical stage of AB was evaluated by Kruskal Wallis test. For correlation analysis of non-parametric continuals and nominal variables the Spearmans' two-tailed test was used.

Results: Our results revealed more than 3.5 times higher levels of sTREM-1 in AB patients (92.3 pg/ml ± 125.6) compared with healthy subjects (25.7 pg/ml ± 9.2; $P = 0.0001$). Higher levels of sTREM-1were found also in patients with exacerbated AB (170.5 pg/ml ± 78.2) compared with non-exacerbated AB patients (59.1 ± 78.2; $P < 0.0001$), patients with respiratory tract obstruction (176.4 pg/ml ± 177.8) than those without obstruction (51.99 pg/ml ± 64.0; $P < 0.0001$) and patients with anti-IgE therapy ($P < 0.0001$). Levels of sTREM-1 correlated with number of leucocytes ($P = 0.002$), and absolute number of neutrophils ($P = 0.001$).

Conclusions: The levels of sTREM-1 in AB patients have not been studied yet, so we cannot compare our Results: . Plasma levels of sTREM-1 are highly elevated in severe forms of asthma, reflect the clinical stage of AB, state of exacerbation, respiratory tract obstruction and correlate with the number of leukocytes, mainly neutrophils. Our results highlight the potential usefulness of the assessment of the soluble form of TREM-1 in plasma of AB patients.

P0814

Inhibition of histamine H1 receptor activity modulates proinflammatory cytokine production of dendritic cells through c-Rel dependent pathway

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Purpose/Objective: Histamine displays diverse effects on immune regulation through four types of histamine receptors (HRs). Among them, type 1 receptor (H1R) plays an important role in allergic inflammation. Dendritic cells (DCs), which express at least three types of HRs, are professional antigen-presenting cells controlling the development of allergic inflammation. However, the molecular mechanisms involved in H1R-mediated NF-kB signaling of DCs remain poorly defined.

Materials and methods: Bone marrow-derived dendritic cells (BM-DCs) were treated with H1R inverse agonist ketotifen to interrupt the basal H1R-mediated signaling. The crosstalk of H1R-mediated signaling and NF-κB pathway was examined by NF-κB subunits analysis using Western blotting and TNF-α promoter activity using chromatin immunoprecipitation assay.

Results: The data showed that blockage of H1R signaling by ketotifen inhibited the proinflammatory cytokine TNF-α and IL-6 production of BM-DCs. H1R specific agonist was able to enhance the TNF-α production, but this overexpression was significantly inhibited by NF-κB inhibitor, suggesting crosstalk between H1R and NF-κB signaling in DCs. After comprehensive analysis of NF-κB subunits, c-Rel protein level was found to be significantly down-regulated in ketotifen-treated BM-DCs, which led to inhibition of the promoter activity of TNF-α.

Conclusions: Our results suggest that c-Rel controls H1R-mediated proinflammatory cytokine production in DCs. This study provides a potential mechanism of H1R-mediated signaling and NF-κB pathway crosstalk in allergic inflammation.

P0815

Inhibition of the co-stimulatory molecule OX40 significantly inhibits inflammation in the chronic house dust mite model of allergic airway inflammation

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Purpose/Objective: Allergic asthma is a chronic inflammatory disease of the lungs that is punctuated by exacerbations caused by infection or exposure to allergens. Using a mouse model that mimics certain aspects of allergic asthma this study investigated how blocking OX40 would effect the recruitment of inflammatory cells to the lung.

Materials and methods: Male balb/c mice were treated with a murine OX40 targeting antibody twice weekly, via subcutaneous injection, from day 0 for 5 weeks. From day 0, mice were sensitized to house dust mite (HDM) extract via intranasal administration 4 times a week for 5 weeks.

At the end of week 5, the study was terminated. Blood was taken from the animals and serum prepared. Animals were cannulated and brochoalveolar lavage (BAL) fluid was collected. Lungs were removed and tissue digest carried out in order to release immune cells within the lung tissue. Inflammatory cell types in the BAL and lung tissue digests were determined using flow cytometry.

Results: Blockade of OX40 resulted in a significant decrease in the number of eosinophils, neutrophils and CD4+ T cells in both the BAL and lung tissue digest compared to vehicle treated animals.

Blocking OX40 significantly reduced the number of IFN-γ (Th1), IL-17A (Th17) and IL-4 (Th2) producing CD4+ T cells in both BAL and lung tissue digests.
Conclusions: Blocking OX40 significantly reduces the infiltration of numerous inflammatory cell types into the lungs of mice. It is also interesting that blocking OX40 has effects on Th17 and Th1 cells. This indicates that blocking OX40 may be beneficial in the treatment of allergic asthma.

P0816
Inhibitory effects of hydrogen sulfide on lung oxidative stress in allergic mice

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Purpose/Objective: Recent studies show that endogenous hydrogen sulfide (H_2S) plays an anti-inflammatory role in the pathogenesis of airway inflammation. This study investigated whether exogenous H_2S may counteract oxidative stress-mediated lung damage in allergic mice.
Materials and methods: Female BALB/c mice previously sensitized with ovalbumin (OVA) were treated with sodium hydrosulfide (NaHS) 30 min before OVA challenge. Forty-Eight hours after antigen-challenge, the mice were killed and leukocyte counting as well as nitrite plus nitrate concentrations were determined in the bronchoalveolar lavage fluid, and lung tissue was analysed for nitric oxide synthase (NOS) activity, iNOS expression, superoxide dismutase (SOD), catalase, glutathione reductase (GR) and glutathione peroxidase (GPx) activities, thiobarbituric acid reactive species (TBARS) and 3-nitrotyrosine containing proteins (3-NT).
Results: Pre-treatment of OVA-sensitized mice with NaHS resulted in significant reduction of both eosinophil and neutrophil migration to the lungs, and prevented the elevation of iNOS expression and activity observed in the lungs from the untreated allergic mice, although it did not affect 3-NT. NaHS treatment also abolished the increased lipid peroxidation present in the allergic mouse lungs and increased SOD, GPx and GR enzyme activities.
Conclusions: These results show, for the first time, that the beneficial *in vivo* effects of the H_2S-donor NaHS on allergic airway inflammation involve its inhibitory action on leukocyte recruitment and the prevention of lung damage by increasing endogenous antioxidant defenses, thus making of H_2S donors a potential new class of therapeutical agents useful for treatment of lung diseases characterized by the presence of inflammatory cells and oxidant/antioxidant imbalance.

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P0817
Intratracheal administration of Fab fragments of an antigen-specific monoclonal antibody suppresses asthmatic responses in mice

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Purpose/Objective: Fab fragments (Fabs) of antibodies maintain the ability to bind specific antigens but lack the binding site for complement (C) as well as the site for binding to receptors on effector cells including mast cells, basophils, and macrophages that play an important role in immune and allergic diseases. In the present study, we investigated whether intratracheal administration of Fabs of a monoclonal antibody (mAb) specific for ovalbumin (OVA) was able to suppress asthmatic responses in mice.
Materials and methods: Asthmatic responses were induced in Balb/c mice either by passive sensitization with anti-OVA pAbs or by active sensitization with OVA followed by intratracheal challenge with the antigen. Fabs prepared by the digestion of an anti-OVA IgG1 (O1−10) mAb with papain were intratracheally administered 30 min before the antigenic challenge. Normal IgG Fabs were used as a control.
Results: Intratracheal administration of O1−10 Fabs markedly suppressed the early and late phases of asthmatic responses caused by passive sensitization with anti-OVA pAbs as well as by active sensitization with OVA when the *in vivo* responses were measured by specific airway resistance (sRaw). Control Fabs failed to affect the asthmatic responses. The significantly reduced number of neutrophils in bronchoalveolar lavage fluids (BALF) as well as in the lung tissue was seen in mice treated with O1−10 FabsO1−10. Fabs were also effective in suppressing asthmatic responses induced by passive sensitization wtih anti-OVA IgE mAb (OE-1). In cotrast, intratracheal injection of intact O1−10 failed to suppress the asthmatic responses. The suppression of asthmatic responses by O1−10 Fabs was associated with significantly higher and lower levels of MMCP-1 and C3a, respectively. *In vitro* studies revealed that the capture of OVA by O1−10 Fabs resulted in prevention of the following binding of intact anti-OVA pAbs or OE-1 to the captured OVA.
Conclusions: Asthmatic responses appear to be downregulated by intratracheal administration of Fabs of an antigen-specific mAb via the mechanism of the formation of antigen and Fab complexes in the airway, resulting in the prevention of antigen and intact antibody binding essential for induction of asthmatic responses.

P0818
Isolation and characterisation of CD8 T cells with receptor specificity for house dust mite allergen, Der p 1

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Purpose/Objective: CD8 T cells have multiple functions in asthma. Tc2 CD8 T cells exacerbate airway inflammation through the secretion of type 2 cytokines, while the Tc1 CD8 T cells may inhibit type 2 airway inflammation and airway hyperresponsiveness via a number of different mechanisms such as the production of type 1 cytokines and perforin-mediated cytotoxicity. Therefore, there exists the potential to target CD8 T cells to for treatment of asthma and other allergic diseases. Our aim was to isolate CD8 T cells specific for the house dust mite protein, Der p 1, and to characterise the T cell receptor (TCR) of these allergen specific T cells.
Materials and methods: DNA vaccination was employed to generate a CD8 T cell response in C57BL/6 mice. Plasmid vector encoding a MHC I epitope from Der p 1 was delivered intradermally by skin tattoo and CD8 T cell responses measured by IFN-g ELISPOT and flow cytometry with MHC I tetramers. T cells isolated from immunized mice were cultured *in vitro* with the antigenic peptide and screened for antigen specificity using MHC I tetramers. TCR genes were identified using 5' RACE amplification.
Results: Following DNA vaccination, 1−1.5% of splenic CD8 T cells were shown to be specific for the Der p 1 epitope. Screening following 4 cycles of re-stimulation showed that 95% the CD8 T cells in the culture were Der p 1-specific. These CD8 T cells killed peptide pulsed EL4 cells and secreted IFN-g and TNF-a. 5' RACE amplification followed by sequencing of TCR genes showed that the cells were clonal with alpha (TRAV7-5*01F) and beta (TRBV5*01F) TCR chains.
Conclusions: We have generated a CD8 T cell response by DNA vaccination and characterized the TCR usage of allergen specific T cells

that recognize an epitope derived from the house dust mite protein, Der p 1. This will facilitate investigation into CD8 T cell regulation of asthma by allergen specific CD8 T cells and the development of a CD8 based vaccine for asthma.

P0819

Isolation, expression and characterization of IgE reactive-portions of high molecular weight glutenin like wheat food allergens

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Purpose/Objective: Wheat can be grown in a wide climatic and geographic range and is one of the most consumed cereals worldwide but it is also an important allergen source. In allergic patients wheat ingestion can lead to urticaria, atopic dermatitis, and gastrointestinal symptoms and to anaphylaxis.

Materials and methods: We isolated IgE-reactive clones from a wheat seed cDNA library with sera from wheat food allergic patients. Two of these clones showed sequence identities with a gene coding for the High molecular weight glutenin x-type subunit Bx7 precursor (HMW Bx7). The IgE reactive sequences were cloned into the *E. coli* expression vectors pMal-c4x or pET17b and expressed as soluble hexaistidine- and maltose-binding-protein-tagged fusion proteins (m43, m82) comprising aa 344−618 and aa 600-795 of the complete protein and as hexahistidne-tagged recombinant protein (43). Dot blot analyses were performed with sera from wheat food allergic patients. Moreover, antibodies were raised against 43 to detect homologous proteins in other allergenic plant food.

Results: The two tested wheat food allergic populations exhibited a similar allergen recognition frequency, 15% of the Greek ($n = 26$) and 10% of the Finnish ($n = 60$) patients showed IgE reactivity to the allergen portions m43 and m82. We detected homologous proteins with the rabbit anti-43 antibodies in extracts from spelt, rye, barley and sunflower seeds. Additional, we found HMW Bx7- related proteins in roll, brown bread, rye bread and in gluten free bread extracts.

Conclusions: In summary, we expressed two IgE reactive parts of the novel wheat food allergen HMW Bx7 as soluble proteins in *E. coli* and showed that they are useful for *in-vitro* diagnostic tests to detect wheat food allergic patients.

P0820

Low levels of serum B-cell activating factor among humans with high IgE reactivity to the nematode *Ascaris*

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Purpose/Objective: The gene *TNFSF13B* encoding the B-Cell Activating Factor (BAFF) is a putative candidate for resistance to *Ascaris* in the 13q33 locus. The polymorphism rs10508198 (G>C) is associated with specific IgE/IgG levels to *Ascaris* and the resistance marker (rABA-1), suggesting that genetic variation in *TNFSF13B* may affect antibody response to *Ascaris*. Mechanisms are unknown but effects are more noticeable in asthmatics. We aim to analyze the serum BAFF levels (sBAFF) among high and low IgE/IgG producers and according to the BAFF-R expression, genotype and asthma status

Materials and Methods: We analyzed 842 subjects (375 asthmatics/467 controls) from Cartagena (Colombia) exposed to *Ascaris*. sBAFF levels were measured by ELISA (R&D Systems). Speci?c IgE/IgG against *Ascaris* and rABA-1 were measured by ELISA. Surface expression of BAFF (CD257) and BAFFR (CD268) was evaluated on monocytes and B cells ($n = 83$) by flow cytometry (Cyan ADP; Dako). Statistical analyses were done on IBM SPSS v20 and GraphPad Prism.

Results: There was an inverse relationship between sBAFF levels and sIgE to *Ascaris*; individuals with high IgE to *Ascaris* had less sBAFF levels (Mean SD: 842 ± 320 versus 788 ± 306 pg/ml in high-IgE, $P = 0.029$). In addition, sBAFF levels were inversely correlated to the surface expression of BAFF-R on $CD19^+$ B cells ($r^2 = -0.53$, $P = 4 \times 10^{-5}$). sBAFF levels did not differ among low and high anti-*Ascaris* IgG producers. Mutant CC homozygotes had more specific IgE to *Ascaris* ($P = 0.03$); increased probability of high IgE levels to *Ascaris* >75th percentile (OR 2.67, 95%CI 1.15−6.18, $P = 0.02$) and less IgG to *Ascaris* ($P = 0.002$). However this polymorphism was not associated with sBAFF levels (Mean SD GG: 831 ± 307 versus CC: 818 ± 282 pg/ml $P = 0.53$). Its effect remains to be evaluated at the transcriptional level since BAFF was not detected in the surface of $CD14^+$ monocytes or B cells. There was no difference in sBAFF levels between asthmatics and controls

Conclusions: sBAFF levels are related to the levels of specific IgE to *Ascaris*, supporting the role of *TNFSF13B*as underlying gene for *Ascaris* susceptibility on the 13q33 locus. We hypothesize that individuals with high IgE to *Ascaris* may harbor variants decreasing gene expression or leading to a hypoactive isoform. Causative polymorphisms and mechanisms remain to be elucidated

P0821

M-cell specific targeting by neuraminidase-functionalized microparticles as a novel oral immunotherapy for food allergy

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Purpose/Objective: There is still no accepted causative treatment available for food allergic disorders. Recently, we demonstrated in a murine study that mucosal M-cell targeting with *Aleuria aurantia* lectin (AAL)-coated Poly (D, L-lactide-co-glycolide) (PLGA) microparticles (MPs) represents a promising oral treatment approach in IgE mediated allergy. Due to its structural similarities with AAL we aimed to analyze neuraminidase (NA) from *Vibrio cholerae* as a novel M-cell-specific functionalization substance and compared NA-, AAL- and wheat germ agglutinin (WGA)-coated, allergen-loaded MPs as a treatment option in food allergy.

Materials and methods: Targeting substances and the functionalized MPs were characterized and tested for their suitability for oral application by Caco2-ELISA, digestion experiments and in a human M-cell like co-culture model. Next, we analyzed the therapeutic

properties of the coated MPs *in vitro* by stimulation of naïve splenocytes and *in vivo* in a BALB/c food allergy model.
Results: NA-coated MPs revealed high binding specificity to α-L-Fucose and Monosialoganglioside 1 (GM1) in cellular ELISA as well as significant enhanced transepithelial transport when M-cells were present in the co-culture model. For investigation of immunogenicity of NA and NA-functionalized MPs we stimulated splenocytes from naïve mice. Cytokine evaluations revealed a significant increase of IL-10 and IFN-γ. Intraperitoneal injection of the food allergen ovalbumin (OVA) followed by two oral challenges induced a strong IgE-mediated, OVA-specific response. After 6 oral treatments either with uncoated or WGA-, AAL- or NA-functionalized OVA-loaded PLGA-MPs, murine immune responses were re-evaluated. Treatment with NA-coated MPs induced increased OVA-specific IgG2a and IgA, whereas IgE and IgG1 levels decreased. In intestinal lavages, we observed a significant reduction of total and OVA-specific IgA levels compared to all other groups. Furthermore, significantly elevated IL-10 and IFN-γ were measured when splenocytes from animals treated with NA-coated MPs were stimulated with OVA.
Conclusions: Based on these results we propose NA to have a beneficial immunomodulatory capacity. NA represents a promising PLGA-MP functionalization substance for treatment of type I food allergy.

P0823
Molecular and immunological characterization of Der p 18, a chitinase-like house dust mite allergen

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Purpose/Objective: The house dust mite allergen Der p 18 belongs to the glycoside hydrolase family 18 chitinases, which can be found in viruses, bacteria, fungi, plants, animals and humans. It is a major allergen in HDM-allergic dogs but its relevance for house dust mite allergic patients has not been studied in detail.

This study aimed to further characterize this allergen on a molecular, structural and immunological level.
Materials and methods: Der p 18 was expressed in *E. coli*, purified to homogeneity and its secondary structure was analyzed by circular dichroism. Der p 18-specific IgG antibodies were produced in rabbits to localize the allergen in mites using immunogold electron microscopy and to search for cross-reactive allergens in other allergen sources (i.e. mites, crustacea, mollusca, insects). IgE reactivity of rDer p 18 was tested with sera from 278 HDM-allergic patients and its allergenic activity was analyzed in basophil degranulation experiments.
Results: Recombinant Der p 18 was expressed as a folded protein which shows cross-reactivity with Der f 18 from *D. farinae* but not with proteins from the other tested allergen sources. The allergen is present in the gut wall of the mites but in contrast to most of the important HDM allergens only to a very small extent in the fecal pellets. Der p 18 reacted with IgE from 6.25% of mite allergic patients from Central Europe, however, the IgE reactivity was considerably higher in HDM-allergic patients suffering from atopic dermatitis (20%). rDer p 18 induced a dose-dependent upregulation of CD203c on basophils from Der p 18-positive patients.
Conclusions: Der p 18 is a genus-specific allergen, which seems to be important for patients with severe disease manifestations (e.g. atopic dermatitis).

P0824
Monitoring of regulatory T cells in children with allergy before and one year after sublingual specific immunotherapy (SLIT) by flow cytometry: preliminary study

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Purpose/Objective: Allergen specific immunotherapy (ASIT) is being used for 100 years and is so far the only specific treatment of pollen/dust allergies. In spite of that, objective laboratory test to monitor the results of ASIT is not available yet. Recently different studies started to highlight the link between regulatory T cells (Tregs) and the outcome of immunotherapy. Some authors demonstrated that upregulation in Tregs may be considered as response-related biomarker for ASIT. The aim of the study was to test the frequency of $CD4^+$ $CD25^{high}$-$FoxP3^+$ $CD127^-$ regulatory T cells in patients with allergy before and one year after SLIT.
Materials and methods: The study involved 30 children with pollen allergy. All patients had characteristic symptoms of asthma, allergic rhinitis and allergic conjunctivitis. PBMCs were stained with monoclonal antibodies (anti-CD25 PE-Cy7, clone M-A251; anti-CD4 PE-Cy5; anti-CD127 PE and anti-FoxP3 Alexa Fluor 488, clone 259/D, Becton Dickinson). The samples were evaluated using flow cytometer Beckman Coulter FC500.
Results: Tregs in peripheral blood were identified as $CD4^+$ $CD25^{high}$-$FoxP3^+$ $CD127^-$ T cells. The number of Tregs is expressed as a percentage of all CD4+ T cells. The percentages of Tregs in both groups of patients (before $n = 25$ and after one year of SLIT $n = 17$) were similar (median 2.21; 2.81, respectively). Twelve patients were analyzed at both time points. Seven patients showed increase of Tregs after treatment and in case of 4 patients Tregs were lower. This results also did not differed significantly (before $n = 12$ median 2.67; one year after treatment $n = 12$ median 2.91).
Conclusions: This preliminary study did not demonstrate that natural Tregs can serve as potential biomarker of effectiveness of SLIT. But definitive conclusions should be drawn after enlargement of study population.

P0825
Naive and memory T cell response to Der p 1 allergen presented by dendritic cells in the presence of by *M. bovis* BCG bacilli in allergic asthma

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Purpose/Objective: Dendritic cells (DC) are crucial in the regulation of Th1/Th2 polarization. Allergy is caused by an excessive development of Th2 profile in immune response to environmental allergens. Many

asthmatic patients develop allergy to Der p 1-major allergen of *Dermatophagoides pteronyssinus* house dust mite. Mycobacteria as strong Th1 inducers are potent candidates to diminish the harmful Th2 profile in allergic disorders.

We asked a question whether in the presence of Der p 1, *Mycobacterium bovis* BCG vaccine and a recombinant BCG producing human IL-18 (rBCGhIL-18) were able to polarize Th2 towards Th1 lymphocyte response of naïve and memory T cells.

Materials and methods: Monocyte-derived dendritic cells (MoDC) were prepared from asthmatic patients who responded to Der p 1 in skin prick test and healthy BCG vaccinated donors. MoDC were stimulated for 24 h with Der p 1 in presence/absence of *M. bovis* BCG or rBCG-IL-18 bacilli. The response of naïve and memory T cells to pulsed MoDC was evaluated by determining the IFN-gamma and IL-5 production by ELISA test.

Results: In response to Der p 1-pulsed MoDC, autologous naïve and memory T cells from allergic patients produced IL-5 significantly more frequently and more intensively as compared with T cells from healthy donors. However, in the group of allergic patient's memory T cells, in response to Der p 1-MoDC released significantly more intensively IL-5 than naïve T cells. BCG and rBCG-hIL-18 bacilli in the presence of Der p 1 presented by MoDC decreased the IL-5 production neither by naïve nor memory T cells.

In contrast to healthy donors, naïve T cells from allergic patients, in response to Der p 1-stimulated MoDC produced significantly more intensively IFN-gamma than identically stimulated memory T cells. Interestingly, BCG and rBCGhIL-18 in the presence of Derp 1 significantly enhanced the intensity of IFN-gamma production by naïve and memory T cells in the group of allergic patients and healthy donors.

Conclusions: The BCG vaccine as well as rBCGhIL-18 was shown to display *in vitro* the ability to induce IFN-gamma production by naïve and memory T cells without the significant IL-5 supression. Thus the potential immunomodulatory role of BCG in allergic asthma seems to be more essential for Th1 enhancement than Th2 supression.

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P0826

Participation of c-Jun N-terminal Kinase 2 (JNK2) in the induction of ovalbumin-induced bronchial asthma

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Purpose/Objective: Bronchial asthma is a chronic inflammatory airway disease characterized by airway hypersensitivity (AHR), inflammatory cell infiltration into the airways including T-helper 2 (Th2) cells, eosinophils, and macrophages. Th2 responses are regulated by several factors including GATA3 and mitogen-activated protein kinases (MAPKs). cJun N-terminal kinase (JNK)2, a member of MAPKs, plays a crucial role in the induction of cell proliferation, apoptosis, and inflammation. In the present study, we examined whether JNK2 is involved in the induction of ovalbumin (OVA)-induced bronchial asthma using JNK2-deficient mice.

Materials and methods: C57BL/6J wild type (WT) and JNK2-deficient mice were sensitized with OVA in PBS intraperitoneally, followed by intranasal administration of OVA. T and B cells from WT or JNK2-deficient mice cells were transferred into Rag2-deficinet mice and immunized with OVA, followed by intranasal administration of OVA. Mice were assayed for AHR, histological section, cytokine levels in bronchoalveolar lavage (BAL) fluid, and antibody production 24 h after the last OVA administration.

Results: JNK2-deficient mice displayed reduced OVA-induced AHR, IgG1 and IgE antibody responses, and airway inflammatory lesion compared with WT mice. Cytokine levels including IL-4, IL-10, and IFN-γ in BAL fluid were also reduced in JNK2-deficient mice relative to WT mice. Transfer of JNK2-deficient T or B cells together with WT B or T cells into Rag2-deficinet mice resulted in diminished inflammatory responses compared with WT T cells plus WT B cells, suggesting that both T and B cells from JNK2-deficient mice are implicated in the pathogenesis of OVA-induced bronchial asthma.

Conclusions: JNK2 activation is involved in the induction of OVA-induced bronchial asthma, probably through participation of both T and B cells.

P0828

Persistence of allergen-specific IgE responses in patients suffering from AIDS

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Purpose/Objective: The infection of CD4+ cells by HIV leads to the progressive destruction of CD4+ T lymphocytes and, after massive reduction of CD4+ cells, to AIDS. To study if HIV-infected patients suffering from AIDS with a severe reduction of CD4+ cells can suffer from symptoms of IgE-mediated allergy and produce allergen-specific IgE antibody.

Materials and methods: In total 69 HIV-infected allergic patients from Zimbabwe were studied. Among these patients, 27 had CD4 counts below 200. Allergy was diagnozed according to case history, physical examination and skin prick testing. Serological analysis of allergen-specific IgE antibodies was performed with an allergen chip, containing 163 purified allergen molecules or with the MAST-CLA® assay, containing a panel of 36 allergen extracts. IgE antibody levels specific for seasonal allergens (Art v 1, Art v 3, Bet v 1, Cup a 1, Cyn d1, Ole e1, Phl p 1) were quantified with ImmunoCAP measurements when follow-up sera obtained at different time points were available. HIV infection was confirmed serologically and the disease was staged clinically. Determination of CD4+ and CD8+T lymphocyte subset numbers were performed by flow cytometry.

Results: The predominant allergic symptoms of HIV-infected patients were IgE-mediated symptoms such as allergic rhinoconjunctivitis and urticaria whereas T cell mediated symptoms (e.g. atopic dermatitis) ceased in patients with very low CD4 counts. In accordance to the clinical symptoms IgE responses specific for house dust mite, grass pollen and moulds were most frequent. ImmunoCAP measurements of IgE levels specific for seasonal allergens indicated that even patients with CD4 counts <200 exhibited boosts of IgE production.

Conclusions: Our results indicate that allergen-specific IgE production and immediate IgE-mediated allergic inflammation do not require a fully functional CD4+ T lymphocyte repertoire.

P0829

Pneumococcal pneumonia suppresses allergy development but preserves respiratory tolerance in mice

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Purpose/Objective: Neonatal colonization with *Streptococcus pneumoniae* (*S. pneumoniae*) is associated with an increased risk for recurrent wheeze and asthma, whereas killed *S. pneumoniae* showed some potential as an effective immunomodulatory therapy.

Materials and methods: We investigated the impact of lung infection with *S. pneumoniae* on the development and maintenance of allergic airway inflammation and respiratory tolerance in mice. BALB/c mice were infected intratracheally with *S. pneumoniae* either before or after tolerance or allergy inducing protocols using ovalbumin (OVA) as model allergen were initiated.
Results: Infection of mice with *S. pneumoniae* prior to sensitization suppressed the development of an allergic phenotype as judged by reduced eosinophils in bronchoalveolar lavage fluids and decreased IgE serum levels and Th2 cytokines, relative to non-infected allergic control mice. In contrast, infection of mice after manifestation of allergic airway inflammation combined with late mucosal re-challenge did not affect the allergic response. Moreover, induction and maintenance of respiratory tolerance to OVA challenge were not altered in *S. pneumoniae*-infected mice, demonstrating that mice remained tolerant to the model allergen and protected from the development of allergic airway inflammation regardless of the time point of infection.
Conclusions: Our results suggest that both, respiratory tolerance induction and maintenance resist an infection with *S. pneumoniae* in mice whereas early bacterial infection might decrease the manifestation of an allergic phenotype.

P0832

Prediction of the development of IgG enhancing allergen-specific IgE binding during birch pollen immunotherapy

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Purpose/Objective: Allergen-specific immunotherapy (SIT), the administration of the disease-causing allergens, is a clinically effective treatment for IgE-mediated allergy. Besides reducing symptoms SIT also affects the course of the disease and has long-lasting effects. One major mechanism of SIT is the induction of allergen-specific IgG antibodies which block IgE recognition of allergens and thus IgE-mediated pathomechanisms. In the case of birch pollen allergy the occurrence of IgG antibodies which can enhance IgE recognition of the major birch pollen allergen, Bet v 1, presumably by a change of the conformation of the Bet v 1 allergen, has been reported.
Materials and methods: Here, we used Bet v 1-specific IgG antibodies induced by immunization of rabbits to study their effect on the binding of birch pollen allergic patients ($n = 18$) IgE to Bet v 1 before Bet v 1-specific SIT. Then we analyzed if the extent of blocking or enhancement of these IgG antibodies on patient's IgE binding to Bet v 1 is associated with alterations of cutaneous sensitivity to Bet v 1 after SIT.
Results: In 16 of the18 patients the inhibition of patients IgE binding to Bet v 1 by the Bet v 1-specific rabbit IgG antibodies was associated with a reduction of cutaneous reactivity to Bet v 1. Interestingly, in two patients who showed no reduction or even an enhancement of skin sensitivity, rabbit anti-Bet v 1 IgG had enhanced patients IgE binding to Bet v 1 in the ELISA test.
Conclusions: Our data thus provide evidence for the development of unfavourable allergen-specific IgE-enhancing IgG antibodies during SIT and indicate that it may be possible predict such responses by serological tests.

P0835

Reactive oxygen species generated by NAD(P)H oxidases in ragweed subpollen particles activate human dendritic cells

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Purpose/Objective: It has previously been reported that ragweed (*Ambrosia artemisiifolia*) pollen grains release subpollen particles (SPPs) of respirable size upon hydration. These SPPs contain allergenic proteins and functional NAD(P)H oxidases. In this study we have examined whether exposure to SPPs induces the activation of dendritic cells.
Materials and methods: To test this assumption, human monocyte-derived dendritic cells (moDCs) were treated with ragweed SPPs and the phenotypic and functional changes in the cells were analysed.
Results: We found that treatment with freshly isolated ragweed SPPs increased the intracellular levels of reactive oxygen species in moDCs. Phagocytosis of SPPs by moDCs, as demonstrated by confocal laser-scanning microscopy, led to an up-regulation of the cell surface expression of CD40, CD80, CD86, and HLA-DQ and an increase in the production of IL-6, TNF-alpha, IL-8, and IL-10. Furthermore, SPP-treated moDCs had an increased capacity to stimulate the proliferation of naïve T cells. Co-culture of SPP-treated moDCs with allogeneic $CD3^+$ pan-T cells resulted in increased secretion of IFN-gamma and IL-17 by T cells of both allergic and non-allergic subjects, but induced the production of IL-4 exclusively from the T cells of allergic individuals. Addition of exogenous NADPH further increased, while heat-inactivation or pre-treatment with diphenyleneiodinium, an inhibitor of NADPH oxidases, strongly diminished, the ability of SPPs to induce phenotypic and functional changes in moDCs, indicating that these processes were mediated, at least partly, by the intrinsic NAD(P)H oxidase activity of SPPs.
Conclusions: Collectively, our data suggest that inhaled ragweed SPPs are fully capable of activating dendritic cells in the airways and initiating both innate and adaptive immune responses.

P0837

Recognition of Phl p 5 B-cell epitope mimic H32 in patients from an Italian allergy clinic

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Purpose/Objective: Sensitization to grass pollen is extremely common in patients suffering from respiratory allergy. Most of them show IgE reactivity to highly cross-reactive group 5 allergens like Phl p 5. We recently could show that a single epitope mimic of Phl p 5, named H32 had profound anti-inflammatory effects in an asthma therapy model in mice.

The aim of the present study was to investigate the prevalence of specific recognition of this mimotope (representing single IgE epitope)

in a representative population, in order to predict its potential impact for immunotherapy of grass pollen allergic patients.
Materials and methods: Mimotope H32 was spotted on an experimental version of the ISAC®-microarray2. Subjects (n = 992) visiting an allergy outpatient clinic in Rome were screened for IgE reactivity using this experimental ISAC. Of these patients 678 were also tested for specific IgG4 to Phl p 5 and/or H32 using the same microarray.
Results: In our study population 28% of patients harbored specific IgE to Phl p 5, whereas 11% reacted to the H32 mimotope. Of the 678 patients that were also screened for specific IgG4 against allergen and mimotope, 45.87% showed IgG4 reactivity to Phl p 5 and 28% towards H32 with low but significant correlation (P = 0.0007).
Conclusions: Although our cohort was biased by the selection criteria (visiting an outpatient allergy clinic), the Phl p 5-IgE-epitope mimicked by mimotope H32 obviously represents an important part of the IgG4 antigenicity of major grass pollen allergen Phl p 5. It may therefore be considered for further investigation into novel therapy strategies of asthma of Phl p 5-allergic patients.

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P0838

Redox-sensitive signaling pathway is upregulated in response to contact sensitizer in human derived monocyte cell

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Purpose/Objective: Allergic contact dermatitis (ACD) is a major cause of occupational skin disease that is caused by delayed-type hypersensitivity responses to antigens (skin sensitizer). A various cells in both skin and immune organs are related to induce ACD. Among them, dendritic cells (DCs) are palying a key role in ACD. But, it is not clear how skin sensitizers induce the activation of DCs. A several studies suggested that Keap1/Nrf2 pathway is involved in DCs activation. Thus, we assessed the activation of Keap1/Nrf2 pathway in U937 cells to skin sensitizers.
Materials and methods: U937 was purchased from ATCC, and was cultured in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycine. Cells were seeded into a 24 well plate (1×10^6 cells/ml). After 24 h, cells were treated at different concentrations. After 24 h incubation with chemicals, cells were cheacked the expression of Nrf2.
Results: Keap1/Nrf2 pathway known to redox-sensitive is affected by redox potential through various mechanisms including direct protein modification, alteration of protein expression, phosphorylation status and transcriptional activity. And Keap1/Nrf2 pathway is a cellular signaling pathway for the detection of endogenous or exogenous electrophiles and is implicated in the regulated response of the cell to pro-oxidant and eletrophilic aggregations. It is suggested that Nrf2 pathway is activated by skin sensitizer in peripheral blood-derived DC, MCF-7 cell or human monocytic cells. Expression of Nrf2 was increased by skin sensitizers in U937 cells.
Conclusions: Our results supported that the Keap1/Nrf2 pathway is activated by skin sensitizer. In addition this study showed that activation of Keap1/Nrf2 pathway is involved in DCs activation.

P0839

Reduced B cell IL-10 in response to LPS in Allergic Asthma patients: an impaired Breg function?

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Purpose/Objective: Allergic asthma is characterized by inflammatory responses against allergens. Murine studies have indicated that IL-10 producing B cells can potently reduce allergic and auto-immune inflammation. Several human regulatory B (Breg) cell subsets have been described with an impaired function in auto-immunity. Those include $CD1d^{hi}$, $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^{+}$ B cells. Here, we aim to study the frequency and function of IL-10 producing B cells in allergic asthma (AA) patients.
Materials and methods: We collected PBMCs from 13 AA patients and sex/age-matched healthy control (HC) subjects. Isolated peripheral B cells were analyzed for the expression of different regulatory B cells markers and, additionally, activated through the B cells receptor or by LPS, followed by a co-culture with endogenous $CD4^{+}$ T cells and Derp1.
Results: B cell phenotype analysis showed equal numbers of $CD1d^{hi}$ B cells, while $CD24^{hi}CD38^{hi}$ B cells were increased and $CD24^{hi}CD27^{+}$ B cells were decreased in AA patients. Subsequently, LPS, but not anti-IgG/anti-IgM stimulation, resulted in less intracellular IL-10 in B cells from AA patients. Subset analysis showed that the reduced intracellular IL-10 in AA patients was only present in $CD24^{hi}CD27^{+}$ B cells, but not in the other two Breg-related subsets. Next, co-cultures of LPS-primed B cells and memory $CD4^{+}$ T cells resulted in more $IL\text{-}10^{+}$ T cells in conditions of HC subjects compared to AA patients, which dependent on the presence of IL-10.
Conclusions: Altogether, this data indicate that $CD24^{hi}CD27^{+}$ B cells from AA patients are lower in number and produce less IL-10 in response LPS, resulting in less $IL\text{-}10^{+}$ T cells. These data suggest that a deficiency in IL-10 producing Breg cells may contribute to allergic asthma.

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P0840

Role of B-lymphocytes in chemical-induced asthma

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Purpose/Objective: The role of B-lymphocytes in chemical-induced asthma is largely unknown. We used a mouse model of chemical-induced asthma and transferred B-lymphocytes from sensitized animals into naïve wild type mice, B-KO mice or SCID mice.
Materials and methods: On days 1 and 8, BALB/c mice were dermally sensitized with 0.3% toluene-2,4-diisocyanate (TDI) on both ears. On day 15, mice were sacrificed and the auricular lymph nodes isolated. B-cells (CD19+) were separated from the whole cell suspension and 175 000 cells were injected in the tail vein of naïve wild type, B-KO or SCID mice. Three days later, the mice received a single oropharyngeal challenge with 0.01% TDI or vehicle (acetone/olive oil) (controls). Airway reactivity to methacholine and total and differential cell counts in the BAL were measured 24 h after challenge. B-cells were characterized for the expression of surface markers and production of cytokines and were labeled with SNARF-1 for tracking.
Results: Wild type mice receiving B-cells from TDI-sensitized mice showed a 3-fold increase in methacholine reactivity and an 8-fold

increase in BAL neutrophils after challenge with TDI, compared to those challenged with vehicle. B-KO mice showed these responses only after transfer of B-cells from TDI-sensitized BALB/c mice. Similarly, transferring B-cells from TDI-sensitized mice in SCID mice also led to a significant increase in methacholine reactivity as well as airway inflammation upon TDI challenge. B-cells of TDI-sensitized mice expressed higher amounts of different surface markers and co-stimulatory molecules and were able to produce significant amounts of cytokines. The transferred B-cells could be visualized in cryosections of the lung.

Conclusions: We were able to passively sensitize naïve mice with B-lymphocytes from TDI-sensitized mice. These mice showed within 3 days an 'asthmatic' response upon challenge with TDI. The response in SCID mice leads to the hypothesis that B-lymphocytes can act without T-cells. The B-lymphocytes were able to generate a mixed B-effector (Be) 1-Be2 response.

P0844

Serum thymic stromal lymphopoietin is a candidate for biomarker of seasonal allergic rhinitis

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Purpose/Objective: Thymic stromal lymphopoietin (TSLP) is an IL-7-related cytokine enhanced under asthma-like conditions, conditioning antigen-presenting cells to orientate the differentiation of T cells towards a Th2 profile, and has been linked to allergic diseases. The present study investigated the relationship between serum TSLP levels and severity of seasonal allergic rhinitis.

Materials and methods: We conducted a randomized, double-blind, placebo-controlled study to determine whether orally administered olopatadine for prophylactic purposes might also be effective for the control of nasal allergy symptoms and serum cytokines in patients with seasonal allergic rhinitis. Amounts of serum TSLP in the patients were measured with a TSLP kit that was purchased from Peprotech. A total of 110 patients with seasonal allergic rhinitis caused by Japanese cedar pollen were randomized to the treatment. The subjects recorded their nasal allergic symptom scores was assessed by the Japanese version of the Rhinoconjunctivity Quality of Life Questionnaire.

Results: Treatment with oral olopatadine significantly suppressed sneezing, rhinorrhea, and nasal congestion. The serum TSLP levels during the peak Japanese cedar pollen season were lower in the olopatadine group than in the placebo group ($P < 0.0005$). The serum TSLP levels in the patients during the peak season were significantly correlated with disease severity (total nasal symptom scores) ($P < 0.005$).

Conclusions: These results suggest that TSLP plays a contributory role in the pathogenesis of seasonal allergic rhinitis and the serum level of TSLP is a candidate of biomarker in seasonal allergic rhinitis.

P0845

Structural basis for the enzymatic activity of the major pollen allergen Phl p 4

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Purpose/Objective: Phl p 4 is one of the major pollen allergens from the timothy grass (*Phleum pratense*) and thus cause of hay fever and other diseases related to pollen allergy. Structural and immunological characterization of the allergen has been conducted to the extent which enables prediction of the major epitopes and a rational design of hypoallergenic derivatives.

However, nothing is known about the enzymatic activity of the allergen and its connection to the allergenicity. Group 4 grass pollen allergens are the only known allergens with the oxidoreductase activity and possess a bicovalently attached FAD. Connection of enzymatic activity and allergenicity of some proteins was shown; most prominent among them are proteases.

Materials and methods: Recombinant allergens are being expressed in the *P. pastoris* expression system. Macromolecular crystallography followed by the X-ray diffraction has been used for the structure determination. Enzymatic assays based on colorgenic substrates and thermofluor experiments have been used for the enzymatic characterization and search for natural substrates. Further experiments with the stopped-flow methods will be used for revealing the redox potential of the FAD. In addition, point mutations are being introduced in order to reveal the role of the key side chains involved in the processes in the active site.

Results: We have revealed that the Phl p 4 catalyses oxidation of glucose, maltose and lactose in presence of an artificial electron acceptor (DCPIP). We have proven that the protein does not react with molecular oxygen, showing that it is a dehydrogenase. We have successfully solved its structure and based on the structural observations prepared active site mutants. Recently these have been successfully expressed in a small scale.

Conclusions: Large scale expression is on the way to produce enough protein for a search for natural substrates. Only after the thorough characterization of its catalytic properties the connection of allergenicity and catalytic properties can be proven or rejected. The active site mutations are planned to bring novel catalytic properties to the protein. If successful, we will prove a principle that far exceeds the interest of only the immunological scientific community.

P0846

Studies on allergic sensitization in the Lithuanian birth cohort

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Purpose/Objective: Cohort studies are of great importance in defining the mechanism responsible for the development of allergy-associated diseases, such as atopic dermatitis, allergic asthma and allergic rhinoconjunctivitis. Although these disorders share genetic and environmental risk factors, it is still under debate whether they are linked or develop sequentially along an atopic pathway. The current study was aimed to determine the pattern of allergic sensitization in the Lithuanian birth cohort ãAlergemol' ($n = 1558$) established as a part of the multi-center European birth cohort ãEuroPrevall'.

Materials and methods: The sensitization pattern in response to different food allergens in the group of infants with food allergy symptoms was studied using allergological methods *in vivo* and *in vitro*. The impact of maternal and environmental risk factors on the early development of food allergy at 6 and 12 months of age was evaluated.

Results: The analysis revealed 1.3% and 2.8% of symptomatic-sensitized subjects at 6 and 12 months of age, respectively. Our study showed that maternal diet, diseases, the use of antibiotics and tobacco smoke during pregnancy had no significant impact on the early sensitization to food allergens. However, infants of atopic mothers were significantly more often sensitized to egg as compared to the infants of non-atopic mothers.

Conclusions: The rate of food sensitization in early age in the Lithuanian birth cohort was different from that reported for other European cohorts.

P0848

The effect of ligand-binding on structure, stability and biological activity of Bet v 1

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Purpose/Objective: In industrialized countries 25% of the population are affected by asthmatic and allergic diseases – still the allergy eliciting mechanism has to be elucidated. Allergenic PR-10 family members strongly bind hydrophobic molecules, suggesting that these interactions could affect their allergenicity. Bet v 1.0101, the major birch allergen, and Na-deoxycholate, a well-known ligand for Bet v 1.0101 with structural similarities to plant steroids, were selected for allergenicity analysis. The influence of ligand binding on conformation, stability, IgE-binding, and thus biological activity of Bet v 1.0101 were investigated in detail.
Materials and methods: Recombinant and heavy isotope labeled Bet v 1.0101 was produced in *E. coli*, purified to homogeneity and characterized physico-chemically. Melting curves of Bet v 1.0101 with and without ligand were recorded by circular dichroic (CD) measurements. IgE epitopes of free and ligand bound Bet v 1.0101 were mapped by NMR spectroscopy using purified human IgE antibodies from serum of a birch pollen allergic patient. Moreover, mediator release assays were performed with either naked or ligand bound Bet v 1.0101 to assess the influence of ligand on biological activity.
Results: CD measurements revealed that ligand bound Bet v 1.0101 has an increased thermal stability. NMR spectroscopy verified that two Na-deoxycholate molecules can bind the Bet v 1.0101 molecule in its hydrophobic cavity. Despite the binding of Na-deoxycholate influenced the 3D structure of Bet v 1.0101, the overall topology of the protein was not altered. The mapped IgE epitopes in free and ligand bound Bet v 1.0101 were identical. Unbound and ligand bound Bet v 1.0101, both induce crosslinking of the Fce receptors on RBL cells but interestingly ligand bound Bet v 1.0101 triggered half-maximal mediator release at lower concentrations.
Conclusions: Ligand binding of Bet v 1.0101 stabilizes the molecule, still the structure and thus IgE epitopes are not affected. IgE antibodies showed higher affinity to ligand bound allergen, inducing a higher crosslinking of the Fce receptors on basophils. These findings lead to the speculation that IgE antibodies were originally produced against Bet v 1.0101 bound to a hydrophobic ligand. However, further studies are needed to gain a better understanding of these mechanisms.

P0849

The IgE-reactivity of Der p 11, the mite paramyosin, differs considerably between certain geographical areas

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Purpose/Objective: More than 20 allergens have been identified in house dust mites (HDM), but little is known about the clinical relevance of the high molecular weight mite allergens. The objective of this investigation was to study the importance of the high molecular weight allergen Der p 11 for HDM allergic patients.
Materials and methods: A synthetic gene coding for Der p 11 was expressed in *E. coli* and rDer p 11 was purified to homogeneity. The secondary structure of the protein was determined by circular dichroism analysis and its localization was determined in the mites by immunogold electron microscopy. The IgE reactivity of rDer p 11 was tested with sera from HDM allergic patients from Europe and Africa in dot-blot assays and the allergenic activity was evaluated by the up-regulation of CD203c expression in allergic patients' basophils.
Results: rDer p 11 is a stable, alpha-helical protein, which shows homology to paramyosins from invertebrates. The allergen is located in the muscle beneath the skin of mite bodies but not in the feces of mites. The IgE-binding frequency of rDer p 11 (Austria 12%, France 5%, Italy 7% and Sweden 11%) and its allergenic activity were low in European populations, though a considerably higher IgE binding frequency of rDer p 11 (36%) was found in Zimbabwe.
Conclusions: Der p 11 might be an important cross-reactive allergen in certain geographical areas.

P0850

The pattern of total/specific IgE levels distribution in Korean by National Health and Nutrition Examination Survey

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Purpose/Objective: Allergies diseases increase in worldwide and become serious problems because they may lead lifetime chronic disease. Allergic sensitization is known to be one of the diagnostic tools for allergic diseases and determination of total/specific IgE is a simple

method for measuring allergic sensitization. Total IgE levels are known to be affected by various factors, such as age, gender, ethnicity, and geographic area, and total IgE values are not always dependent on specific IgE elevation. Thus, cutoff values of total IgE can vary among different environmental conditions. Therefore, we evaluated the distribution of total and specific IgE levels by the age and gender using population based studies like preschool children survey and National Health and Nutrition Examination Survey in Korea.

Materials and methods: Serum specific IgE tests for 3 allergens (*D. farinae*, Cockroach, Dog) and total IgE tests were performed on 2342 people above age 10 in general population as well as 510 of preschool children aged 3−6 by using UniCAP System. Atopy was defined as a positive specific IgE response (≥0.35 kU/l) to at least one of the allergens. Allergic diseases were defined as a history of asthma, allergic rhinitis or atopic dermatitis diagnosed at any point in the lifetime.

Results: Rapid increase of total IgE was observed in preschool age group and it decreased in the age group of 10−40. Then it increased again in the age group above 50. The most prevalent sensitized allergen was *D. farinae* throughout the entire age group.

Conclusions: Total and specific IgE levels showed various range with age and gender. It was supposed that income and education level may affect total IgE levels. Atopic prevalence and total IgE can be related with allergic diseases. Further investigation is needed with broad ranges of age to give clues for diagnosis and treatment of allergic disease.

P0853

The role of mast cells, IL-13 en TRP channels in a mouse model of chemical-induced airway hyperresponsiveness

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Purpose/Objective: The mechanisms of occupational asthma caused by chemicals are still not completely understood. We used an established mouse model of chemical-induced asthma to examine the role of the neurogenic system, as well as the role of IL-13 and mast cells by using different knock-out mice.

Materials and methods: On days 1 and 8, wild type C57Bl/6 mice and mice deficient in IL-13, transient receptor potential (TRP) V1, TRPA1 or mast cells received dermal applications of 1% toluene-2.4-diisocyanate (TDI) or vehicle (acetone/olive oil) on both ears. On day 15, the mice received a single intranasal challenge with 0.1% TDI or vehicle. In a second experiment, TDI sensitized wild type C57Bl/6 mice received an intraperitoneal injection of the NK1R antagonist RP67580 (1 $\mu g/\mu l$) prior to the challenge. Airway reactivity to methacholine, lung inflammation, lymphocyte subpopulations in the draining auricular lymph nodes and total serum IgE were assessed 24 h after the challenge.

Results: IL-13, TRPV1, TRPA1 and mast cell deficient mice showed a significantly lower airway hyperreactivity compared to wild type mice, without any sign of lung inflammation, 24h after TDI challenge. Treatment with the NK1R antagonist also resulted in a significant decrease in airway hyperreactivity. In the auricular lymph nodes T-helper cells, T-cytotoxic cells and B-cells were significantly lower in mast cell deficient and IL-13 deficient mice, compared to wild type mice.

Conclusions: These results indicate the importance of IL-13, TRPA1 and TRPV1 channels and mast cells in the development of immune-mediated bronchial hyperreactivity.

P0854

Transfer of allergen-expressing bone marrow under costimulation blockade leads to robust long-term tolerance in IgE-mediated allergy

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Purpose/Objective: Molecular chimerism induces tolerance in diseases for which the culprit antigens have been well characterized as is the case in IgE-mediated allergy. While robust and durable tolerance was established towards the grass pollen allergen Phl p 5 with this strategy, it requires myelosuppressive conditioning of the recipient (i.e. irradiation). Here we investigated, if an acceptable recipient conditioning without irradiation can be developed.

Materials and methods: Towards this end we generated a novel transgenic mouse (on the Balb/c background) expressing the major grass pollen allergen Phl p 5 ubiquitously on the surface. This transgenic mouse served as cell donor. Balb/c recipients were conditioned with either 1 Gy total body irradiation or no irradiation in addition to a short term treatment of rapamycin (day-1, -0, -2) and costimulation blockade (anti-CD40L, d0 or CTLA4-Ig, d2 1 mg each). 15×10^6 BMC harvested from the Phl p 5-transgenic mouse (Balb/c background) were transferred iv into conditioned recipients. Subsequently, mice were challenged several times with splenocytes (+AlOH3) of transgenic mice and control allergen (Bet v 1, weeks 4, 7, 10 after BM transplantation [BMT]). Tolerance was analyzed by ELISA for allergen-specific isotypes and T-cell proliferation assays. Airway hyperresponsiveness was determined by whole body plethysmography.

Results: Minimally irradiated recipients (1 Gy) developed persistent and stable macrochimerism (chimerism levels e.g. 35w postBMT): 35% T cells 25% myeloid cells) ($n = 19$). In contrast non-irradiated recipients were not macrochimeric at any time point ($n = 20$). Strikingly, however, Phl p 5-specific IgE was not detectable both in chimeric mice (20/20) and in non-irradiated non-chimeric recipients (e.g. w20 post BMT 7/10). High levels of Bet v 1-specific IgE developed in both groups. Furthermore Phl p 5-specific T-cell proliferation was not detectable at the end of follow up (w35 post BMT). Additionally preliminary data reveal no airway hyperresponsiveness in chimeric recipients (6/6) compared to untreated sensitized controls ($n = 3$).

Conclusions: Transfer of allergen-bearing BMC with costimulation blockade and short course rapamycin leads to robust tolerance without myelosuppressive recipient conditioning. These data suggest that preventive cell-therapy is feasible with clinically acceptable toxicity.

P0855

Vaccination with carrier-bound peptides from the major birch pollen allergen Bet v 1 lacking allergen-specific T cell epitopes reduces Bet v 1-specific T cell responses

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Purpose/Objective: Recently a strategy for the development of safe allergy vaccines has been developed, which is based on the hapten-carrier principle. Allergen-derived peptides lacking IgE-reactivity and allergen-specific T cell epitopes were coupled to a carrier molecule,

which provided T cell help for the induction of a protective allergen-specific IgG antibody response. These vaccines should increase safety of immunotherapy by reducing IgE- as well as T cell-mediated side effects. In this study we investigated in a mouse model whether vaccination with carrier-bound peptides from the major birch pollen allergen Bet v 1 lacking allergen-specific T cell epitopes has influence on Bet v 1-specific T cell responses.

Materials and methods: Three Bet v 1-derived peptides, devoid of Bet v 1-specific T cell epitopes, were coupled to KLH and adsorbed to aluminium hydroxide to obtain a Bet v 1-specific allergy vaccine. Groups of BALB/c mice where immunized with the peptide vaccine before or after sensitization to Bet v 1. Bet v 1- and peptide-specific antibody responses were analyzed by ELISA. T cell responses to Bet v 1, KLH, and the peptides were studied in proliferation assays.

Results: Prophylactic and therapeutic vaccination with carrier-bound Bet v 1 peptides induced a Bet v 1-specific IgG_1 antibody response without priming/boosting of Bet v 1-specific T cells. Both, prophylactic and therapeutic vaccination of mice with the peptide vaccine suppressed Bet v 1-specific T cell responses. Thus, vaccination with carrier-bound allergen-derived peptides lacking allergen-specific T cell epitopes induced allergen-specific antibodies capable of suppressing allergen-specific T cell responses.

Conclusions: Carrier-bound allergen peptides inducing allergen-specific blocking IgG may be useful for the treatment and prevention of allergy.

P0856

Vitamin D drives alpha-1-antitrypsin expression in human lymphocytes and predicts levels in airway lavage

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Purpose/Objective: Epidemiological data associate vitamin D sufficiency with pulmonary health. This study investigated novel properties of vitamin D relevant to airway immune homeostasis, beyond well-documented effects on antimicrobial pathways and regulatory T cells. Alpha-1-antitrypsin (α-1-AT) protects the airway from neutrophil elastase, and additionally exerts immunomodulatory properties; its regulation by vitamin D was therefore examined.

Materials and methods: α-1-AT mRNA and protein expression were analyzed by qPCR and ELISA in peripheral leukocytes from healthy blood donors, human airway-derived populations, and clinical samples (peripheral lymphocytes, bronchoalveolar lavage and matched serum) from adult and paediatric asthmatics and controls. Ethical approval and patient/parental consent were obtained.

Results: SERPINA1 (encoding α-1-AT) was among the genes most highly upregulated by vitamin D treatment (1α,25-dihydroxyvitamin D3, 10^{-7}M) in peripheral CD4 T cells *in vitro*. ELISA confirmed vitamin D-induced α-1-AT secretion by CD4 and CD8 T cells, but not myeloid populations, which secreted comparable α-1-AT when LPS-activated. Human airway-derived T cells also responded to vitamin D to markedly upregulate SERPINA1. In contrast matrix metalloprotease-9, which degrades α-1-AT, was significantly inhibited in total airway cells. In a small number of adult asthmatics, oral vitamin D treatment (1α,25-dihydroxyvitamin D3, 0.5 μg/day) promoted peripheral T cell SERPINA1 expression *in vivo*, and in a larger paediatric cohort, serum vitamin D (25-hydroxyvitamin D3) correlated highly with airway α-1-AT.

Conclusions: These studies identify α-1-AT as a vitamin D-regulated molecule in human peripheral and airway-resident lymphocytes. Vitamin D status appears also to predict α-1-AT levels in the human airway. This finding has clinical implications, since α-1-AT regulates elastolysis in the airway. The immunological implications, given the immunomodulatory properties of α-1-AT, are now under further active investigation.

P0857

Wasp venom activates DC in a TLR-4-dependent way, resulting in subsequent histamine release by circulating basophils

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Purpose/Objective: The activation of dendritic cells (DC) via Toll-like receptors (TLR) plays a decisive role in shaping the outcome of primary immune responses. Recently, others have suggested that allergens may interact with TLR signal transduction, thereby inducing allergic inflammation. Hymenoptera venoms are important allergens that can elicit both local and systemic allergic reactions, including life-threatening anaphylaxis. In this study, we anticipate elucidating possible mechanisms of innate sensing of wasp venom by DC, and investigate the role of TLR.

Materials and methods: Monocyte-derived dendritic cells were *in vitro* generated from healthy controls. Next, phenotype, cytokine profile, and basophil stimulatory potential of DC upon stimulation with wasp venom was investigated. In order to evaluate the mechanism by which wasp venom triggers a pro-inflammatory innate immune response, DC were treated with a Toll-interleukin 1 Receptor (TIR) domain-containing Adapter Protein (TIRAP) inhibitory peptide.

Results: Using HEK293-derived cells that stably express human TLR genes (TLR2, 3, 4, 5, 7, 8, and 9), we identified that wasp venom (Pharmalgen) was recognized by TLR4. Furthermore, *in vitro* generated DC displayed an activated phenotype upon wasp venom stimulation, as evidenced by induction of the secretion of an array of both Th1 and Th2 pro-inflammatory cytokines. A TIRAP inhibitory peptide diminished the ability of DC to secrete pro-inflammatory cytokines. Importantly, conditioned medium of wasp venom stimulated DC induced pronounced histamine release by circulating basophils as compared to conditioned medium of control DC.

Conclusions: Here we show possible involvement of TLR4 in sensing wasp venom. Indeed, wasp venom induced a pro-inflammatory response by DC, in part by direct activation of TLR4. Furthermore, we provide evidence that activation of DC by wasp venom results in subsequent histamine release by basophils. We hypothesize that loss of control of DC-basophil crosstalk contributes to inflammatory responses in patients with hymenoptera venom allergy. For this, we are currently investigating if the proposed mechanisms can be affected by hymenoptera venom immunotherapy.

P0858

Whole milk and butter; clinically significant anti-inflammatory effects

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Purpose/Objective: Recurrent respiratory tract symptoms are a common phenomenon in children. They include wheezing, coughing, rasping and the common cold. Whole milk and natural butter contain fat soluble vitamins, among others vitamin A and E, but also vitamin C, all vitamins with anti-oxidative capacities. We hypothesize that with a dietary change to these dairy products, respiratory tract symptoms will decrease.

Materials and methods: Children aged between 1–6 years with recurrent respiratory tract symptoms were included. The intervention consisted of a dietary advice of daily whole milk/yoghurt and natural butter for 3 months. The control group could continue their usual semi skimmed milk and low fat margarine consumption as before. Respiratory symptoms were collected with 3-month diaries.

Results: Of the 43 patients were included in the intervention group, 25 in the control group. After 3 months of following the dietary advice, the children were significantly less rasping; from 16.3 (SEM = 1.5) to 6.0 (SEM = 1.0) days a month ($P < 0.000$). Also, days with fever (4.5–1.4 days a month, $P < 0.000$), coughing (17.6–10.1 days a month, $P < 0.000$) and common cold decreased significantly (17.2–11.7 days a month, $P = 0.007$) in the intervention group. Wheezing was not affected by the dietary advice (4.5–3.2 days a month, $P > 0.05$). The body mass index was not altered after the change in diet ($P = 0.660$).

Conclusions: Consumption of whole milk and butter for 3 months significantly decreases respiratory tract complaints in children. Wheezing, the only symptom reactive to medication (bronchodilatators), was not affected by the dietary advice.

We conclude that a simple dietary change can improve respiratory tract complaints without side effects significantly.

Poster Session: DC, MHC, T Cells and Disease

P0859

12/15-lipoxygenase-mediated lipid oxidation regulates maturation of dendritic cells

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Purpose/Objective: The enzyme 12/15-lipoxygenase (12/15-LO) mediates lipid oxidation and its expression is restricted to different cells of the monocytic lineage. To further determine the physiological role of this enzyme, we studied a potential role of 12/15-LO during the regulation of DC maturation and the shaping of the consecutive T-cell response.
Materials and methods: We analysed bone marrow-derived DCs of 12/15-LO-deficient mice with regard to their maturation status and cytokine profile *in vitro*. Moreover we examined the consequences of 12/15-LO-deficiency on autoimmune diseases like EAE.
Results: Differentiated bone marrow-derived DCs highly expressed 12/15-LO. These cells were enriched in 12/15-LO-specific phosphatidylcholine-derived oxidation products (oxPAPC). Deletion of 12/15-LO resulted in an increased expression of co-stimulatory molecules. Likewise, 12/15-LO$^{-/-}$ DCs displayed an altered cytokine profile with an elevated expression of the Th17-promoting IL-23 subunit p19. Addition of the 12/15-LO product oxPAPC, in turn, interfered with the maturation of DCs and the expression of p19. After initiation of a specific immune response 12/15-LO-deficient mice displayed an altered cytokine expression profile within their lymph nodes and showed an increased differentiation of Th17 T-cells. In accordance with these findings, the Th17-driven disease model of experimental autoimmune encephalomyelitis (EAE) exacerbated in 12/15-LO$^{-/-}$ mice.
Conclusions: Together these data identify 12/15-LO as a major source of bioactive phospholipid oxidation products in DCs and indicate a central role for enzymatic lipid oxidation during the regulation of the maturation status of DCs and the initiation of an adaptive immune response.

P0860

Activation of invariant natural killer T-cells in periodontitis lesions

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Purpose/Objective: Periodontitis is one of the most prevalent human inflammatory diseases. The major clinical phenotypes of this polymicrobial, biofilm-mediated disease are chronic and aggressive periodontitis, the latter being characterized by a rapid course of destruction that is generally attributed to an altered immune-inflammatory response against periodontal pathogens. Whereas aggressive periodontitis has been causally linked to infection with *Aggregatibacter actinomycetemcomitans* (A.a.), the major pathogen in chronic periodontitis is *Porphyromonas gingivalis* (P.g.). Still, the biological basis for the pathophysiological distinction of the two disease categories has not been well documented yet. Type I natural killer T (NKT) cells are a lymphocyte subset with important roles in regulating immune responses to either tolerance or immunity, including responses against bacterial pathogens. Here, we delineate the mechanisms of NKT cell activation in periodontal infections.
Materials and methods: Dendritic cells (DC) were generated from murine bone marrow by 6-days-culture with GM-CSF. NKT cells were isolated from murine livers. DC were challenged with A.a. or P.g. for 24 h. before they were used as stimulator cells for NKT cells for 24–48 h. before cytokines were tested by ELISA. Inflammatory responses in DC upon challenge with A.a. and P.g. were analyzed by Illumina microarray analysis of *in-vitro* infected DC. *De novo* glycosphingolipid turnover in bacterial challenged DC was tested using a fluorescent alpha-galactosidase A substrate and flow cytometry.
Results: We show a selective infiltration of type I NKT cells in aggressive, but not chronic periodontitis lesions *in vivo*. Murine DCs infected with A.a. triggered a type I interferon response followed by type I NKT cell activation. This stimulation was dependent on the expression of CD1d molecules and signaling via Toll-like receptors in DCs. In contrast, infection with P.g. did not induce NKT cell activation. The stimulatory capacity of DCs on NKT cells required DC self-activation through binding of type I interferon receptors and beta-interferon secretion. Addition of exogenous beta-interferon to P.g.-infected DCs restored the ability to activate NKT cells.
Conclusions: Our study provides a conceivable biological distinction between the two periodontitis subforms and identifies factors required for the activation of the immune system in response to periodontal bacteria.

P0861

Activation of Th17 pathway in potentially prediabetic first degree relatives and type 1 diabetic patients after exposure of diabetes-associated autoantigens

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Purpose/Objective: Type 1 Diabetes (T1D) appears to be mainly a Th mediated autoimmune process however its complexity is still not fully understood. New discovery of molecule or pathways converge in beta-cell destruction is a potential target for future therapeutic avenues. In our study we analysed the effect of diabetes-associated autoantigens on peripheral blood mononuclear cells (PBMC) by gene expression microarray and quantitative RT-PCR with the aim to identify prediabetes-associated cell processes.
Materials and methods: PBMC were sampled from first degree relatives of T1D patients (FDR), T1D patients and healthy controls (HC). Isolated PBMC were cultivated with and without mixture of diabetogenic autoantigens (GAD65, IA2 and proinsulin derived peptides) for 72 h. Then co-culture total RNA was isolated and gene expression arrays were done on 43 probands by high density Phalanx array. We saw the significant changes in Th-17 pathway. In the next step we analysed 53 probands by qRT-PCR TaqMan Gene Expression assays (Life Technologies) focusing on known players of this pathway (RORC, STAT3, IL-17A and TGFβ).
Results: We observed higher expression of IL-17A at T1D patients compared to FDR autoantibody negative ($P = 0.035$). Interestingly we saw significantly higher gene expression of TGFβ ($P = 0.01$) and

STAT3 ($P = 0.007$) comparing T1D patients and FDR autoantibody positive to HC. Gene expression of RORC had similar trend as the TGFβ and STAT3 but the difference was not significant ($P > 0.05$).
Conclusions: TGFβ cytokine is responsible for Th17 and Treg lymphocytes balance. In our study higher expression of TGFβ is associated with Th17 pathway change which is in contrast to previous study. Better understanding of this step can contribute developing immune-based intervention therapy.

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P0862

Aggravation of atherosclerosis by MHC class II antigen deficiency is associated loss of regulatory T cells and expansion of pro-inflammatory Ly-6hi monocytes

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Purpose/Objective: Adaptive immunity in atherosclerosis includes both pro- and anti-inflammatory immune responses. It has been assumed that atherosclerosis involves a loss of tolerance against modified self-antigens generated in response to hypercholesterolemia and that presentation of such antigens on MHC class II molecules lead to activation of pro-inflammatory Th1 cells. Furthermore, anti-inflammatory Tregs have been shown to reduce atherosclerosis development indicating that it might be possible to modulate the balance of pro- and anti-inflammatory responses. We have previously shown that immunizations of hypercholesterolemic mice with an Apo B-100 peptide based vaccine have the ability to reduce atherosclerosis and at the same time increase Treg frequency. Moreover, in hypercholesterolemic mice immunizations with the adjuvant Alum alone also reduce atherosclerosis and increase Tregs which is associated with a capture of oxLDL antigens.
Materials and methods: To further study the role of adaptive immune responses in atherosclerosis we have used hypercholesterolemic mice lacking MHC class II ($ApoE^{-/-}$ $MHCII^{-/-}$).
Results: As expected $ApoE^{-/-}$ $MHCII^{-/-}$ mice had reducedlevels of CD4+ cells, including Tregs as well as low levels IgG and IgM and Th1 and Th2 cytokines in plasma. CD115+ monocytes were reduced in spleen as well as the plasma levels of TNF-a, IL-1b and IL-6 indicating reduced systemic inflammation. In spite of this, $ApoE^{-/-}$ $MHCII^{-/-}$ mice had significantly more atherosclerosis as assessed both by en face Oil Red O staining of the aorta ($4.7 \pm 2.9\%$ versus $1.9 \pm 1.3\%$; $P < 0.01$) and cross-sectional area of subvalvular lesions ($7.7 \pm 2.2 \times 10^5$ versus $4.6 \pm 2.8 \times 10^5$ mm^2; $P < 0.05$). Moreover, macrophage accumulation in lesions was significantly increased ($44.8 \pm 8.0\%$ versus $24.8 \pm 7.8\%$ MOMA-2 stained area; $P < 0.001$). Furthermore, pro-inflammatory Ly-$6c^{hi}$ monocytes were increased in the spleen.
Conclusions: The present observations unexpectedly show that the net effect of MHC class II-dependent antigen presentation in atherosclerosis is athero-protective. The combination of reduced frequency of protective Tregs together with the increase in disease promoting Ly-$6c^{hi}$ monocytes can be the cause of the increased atherosclerosis in this animal model.

P0863

Allergic diseases exhibit various TREC number in $CD4^+$ and $CD8^+$ T-cell subpopulations

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Purpose/Objective: We used T-cell receptor excision circles (TREC) to investigate the mechanisms for replenishment of the peripheral T cell poll in allergic diseases. The study included 26 patients with atopic dermatitis (AD), mean age 26 ± 9.7 years and 50 patients with different form of bronchial asthma (BA), mean age 43 ± 13.8 years. Since the number of TREC decreased with age, for each group of patients was selected a group of 22 healthy people. The average age of donors was 27 ± 4.6 years for patients with AD, and 43 ± 12.3 years for patients with BA, respectively.
Materials and methods: TREC number was measured by quantitative Real-time PCR technique in pure populations of CD4+ and CD8+ lymphocytes using specific primers, probe and standard. CD4+ and CD8+ T cells were sorted by flow cytometer FACSAria (Beckton Dickinson) using fluorescent-conjugated anti-CD3 and anti-CD4 antibodies. Reanalysis of the isolated subsets showed that the purities were at least 95%.
Results: Patients with AD had reduced TREC levels in CD4+ and CD8+ cells, but absolute TREC content in this populations was the same as in normal controls. Also absolute number of CD4+ and CD8+ cells was increased in AD. In contrast, patients with BA had no dissimilarities in the TREC number within both CD4+ and CD8+ T-cell subpopulations from healthy volunteers, as there were no significant differences in TREC content depending on the form of asthma and IgE levels. But a large variation was observed in TREC level according to the duration of the disease. Adults suffering from asthma about 1 year had increased absolute and relative numbers of TREC regarding to healthy controls, whereas in patients with disease duration >1 year they were comparable with donor values. Both groups of patients with the different disease duration had enlarged absolute number of CD4+ T cells and slightly extended absolute number of CD8+ T cells.
Conclusions: Thus, men suffering from AD have a normal thymic function, and TREC 'dilution' caused by a high rate of the peripheral T cell proliferation under conditions of the chronical antigen stimulation. In the onset of BA peripheral expansion and thymopoiesis are imbalance, and thymic emigrants make a grater contribution to the replenishment of the T cell pool. Patients suffering from asthma more than 1 year received additional corticosteroids therapy, that may be related to the turning of TREC level to the normal range in these people.

P0864

Altered co-stimulatory phenotype of Nrf2 knockout dendritic cells is not a result of elevated ROS levels

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Purpose/Objective: Dendritic cells (DCs) are antigen-presenting cells which play a pivotal role in adaptive immune responses. Cellular redox state has an important impact in DC immune function. The redox homeostasis in DCs is mainly controlled through the activity of the transcription factor, Nrf2. Our previous findings revealed that loss of Nrf2 resulted in enhanced co-stimulatory molecule expression and altered immune functions in dendritic cells. In the current study, we

investigated whether the loss of Nrf2 alters basal ROS levels in DCs. Additionally; we tested whether the increase in the co-stimulatory molecule expression is directly due to any changes in ROS levels in Nrf2 knockout DCs.
Materials and methods: Bone marrow-derived immature DCs from Nrf2 knockout and Nrf2 wild type mice were assayed for ROS levels by flow cytometry. ROS levels were lowered by treatment with vitamins C and E (ROS scavengers) followed by evaluation of co-stimulatory molecule expression and ability of DC to stimulate T cell proliferation.
Results: Nrf2 knockout DCs have higher levels of basal ROS compared to Nrf2 wild DCs. Vitamin C & E treatment reduced the levels of ROS in Nrf2 knockout DCs to levels comparable to that of the wild type. However, elevated co-stimulatory molecule (MHCII and CD86) expression of Nrf2 knockout DCs could not be reversed by vitamins treatment.
Conclusions: Our results indicate that the increase in ROS levels in Nrf2 knockout DCs does not contribute to the increase in the co-stimulatory molecule expression.

P0865

Amelioration of experimental autoimmune encephalomyelitis by quinoline-3-carboxamide ABR-215757 is due to target of T cell activation

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Purpose/Objective: ABR-215757 (5757) is a quinoline-3-carboxamide (Q-compound) currently in clinical development for systemic lupus erythematosus (SLE). Q-compounds have shown efficacy in different autoimmune diseases where they ameliorate the disease. However, the mechanism of action is still unknown.
Materials and methods: Experimental autoimmune encephalomyelitis (EAE) is a T cell dependent mouse model of multiple sclerosis. We have used this model to try to understand the mechanism underlying the amelioration by 5757.
Results: Of the 5757 effectively reduces induction of EAE. This mechanism is an early effect since treatment with 5757 during the first 5 days of the disease also reduces EAE development. Influx of T cells and myeloid cells to the brain is significantly reduced by 5757. EAE is a T cell dependent disease and the activation of T cells occurs early in the peripheral lymphoid organs. T cells show a reduced proliferation in the presence of 5757. The effector function of the T cells is affected since T cells from treated animals show a reduced production of IFN-γ and IL-17. The *ex vivo* antigen specific recall response is also heavily reduced in cells from 5757 treated animals.
Conclusions: Taken together, this suggests that 5757 treatment affects T cell activation and maturation of effector cells, thereby reducing EAE development.

P0866

Analysis of *in vitro* T-cell responses of PBMCs from patients with pemphigus vulgaris and healthy controls using desmoglein 3 peptides and peptide conjugates as antigens

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Purpose/Objective: Pemphigus vulgaris (PV) is a rare, life-threatening autoimmune bullous skin disorder. The presence of autoreactive serum antibodies (IgG1 and IgG4) against desmosomal adhesion proteins, desmoglein 1 (Dsg1) and desmoglein 3 (Dsg3), results in mucosal lesions, mucocutaneous blisters and erosions. Autoantigen-specific T-cells also play a crucial role in the initiation and perpetuation of Dsg3/Dsg1-specific T-cell responses. In PV the CD4+ autoreactive T-cells recognize specific immunodominant regions from proteins Dsg3 and/or Dsg1. Patients show autoimmunity only to protein Dsg3 in the early stage of disease, while at the later stage, non-crossreactive immunity appears to both proteins Dsg3 and Dsg1. Protein Dsg3 is the primary target antigen in PV.
Materials and methods: In our study T-cell epitope regions have been selected and represented by synthetic oligopeptides. Synthetic peptides with distinct *in vitro* T-cell response on the peripheral blood monomorphonuclear cells (PBMC) isolated from PV patients have been chosen for conjugation. Conjugating the T-cell peptides to carrier molecules can increase their *in vitro* stimulating activity on PBMC. The selected peptides were conjugated to oligotuftsin [$(TKPKG)_4$] carrier. These peptides and peptide conjugates were used for *in vitro* stimulation of the PBMC from PV patients and healthy controls. After 24 and 48 h of incubation the produced IFN-γ was determined from the supernatants by ELISA.
Results: The synthetic Dsg3 peptides and peptide-oligotuftsin conjugates induced different *in vitro* IFN-γ production rate on PBMC obtained from PV patients and healthy controls determined by ELISA. The *in vitro* stimulatory activity of oligopeptides and peptide-conjugates will be discussed.
Conclusions: Our approach identified promising oligopeptide conjugate candidates as synthetic antigens.

P0867

Analysis of peripheral blood CD56+ and CD57+ NK and NKT cells subsets in pregnant women

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Purpose/Objective: Increased peripheral blood natural killer (NK) cells have been associated with unexplained reproductive failures. Data about the functional role of different NK cells and natural killer T cells (NKT) subsets during pregnancy are contradictory. The aim of this study was to investigate the differences in the relative distribution and the phenotype characteristic of peripheral blood subpopulations of NK and NKT cells in pregnant women with unexplained pregnancy loss compared to healthy pregnant women.
Materials and methods: Fifteen women in the first trimester of pregnancy with a history ofpregnancy loss (Group I), 12 healthy women in the first trimester of pregnancy (Group II), and 27 non-pregnant fertile women with a history of successful pregnancies (Controls) were included in this study. Five-color flow cytometry was used to assess the percentage of peripheral blood $CD3^-$ CD56+ cells (CD56-NK), $CD3^-$ CD57+ cells ($CD57^-$NK), $CD3^+$ CD56 + ($CD56^-$NKT, $CD3^+$ CD57+ cells ($CD57^-$NKT).
Results: CD56-NK and CD57-NK as a mean percentage of total peripheral lymphocytes were significantly decreased in the healthy pregnant women (8.97 ± 3.09 and 3.42 ± 1.75, respectively) compared to controls (12.64 ± 4.4 and 5.73 ± 2.63) and to Group I (13.32 ± 4.92 and 6.22 ± 2.81). Pregnant women with a history of pregnancy loss had significantly higher percentage of CD56-NK and CD57-NK (13.32 ± 4.92 and 6.22 ± 2.81) than those of Group II (8.97 ± 3.09 and 3.42 ± 1.75), but no differences were found when compared to controls (12.64 ± 4.4 and 5.73 ± 2.63). CD56-NKT percentage was nearly equal in all study groups (Group I − 5.66 ± 3.08, Group II − 4.64 ± 2.97, Controls − 4.50 ± 2.71). CD-57-NKT was significantly decreased in healthy pregnant women (6.02 ± 4.35) compared to

Group I (10.30 ± 5.67) and decreased without reaching statistical significance compared to Controls (9.00 ± 5.33).
Conclusions: On the basis of these results we may suppose that CD57 positive NKT and NK subsets may be involved in the immunological phenomenon of pregnancy and in the immune response disturbances in risk pregnancy.

P0868

Antigen presenting cells are important to modulate CD4+ T cells in renal ischemia and reperfusion injury

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Purpose/Objective: Ischemia and reperfusion injury (IRI) is an acute inflammatory response considered to be the main cause of acute renal injury in kidneys. Antigen presenting cells (APC), as dendritic cells (DC) and macrophages, have an important role in IRI, although a few is known about their functionality and their possible role in T cell activation in this model. In this way, we investigated whether APC were involved in IRI and their participation in T cell modulation in this model.
Materials and methods: We used C57Bl/6 and C57Bl/6$^-$ CD11c-DTR mice to perform the ischemia and sham group as control. In order to deplete, we used a clodronate protocol to deplete phagocytes or the CD11c-DTR mice diphtheria toxin (4 ng/g of mice) to deplete CD11c+ cells.
Results: We observed that after 24 h of reperfusion, urea (>250 mg/dl) and creatinine (>1 mg/dl) levels were increased in ischemic group, indicating injury. No difference of DC numbers were observed in the kidney and draining lymph nodes, however, DC from ischemic group were more activated, presenting more pronounced expression of CD86. We then used two models of depletion of APC, as mentioned above. Both protocols resulted in increased levels of urea (>300 mg/dl) and creatinine (>2 mg/dl) in the serum of APC depleted mice. In order to investigate the influence of DC in CD4+ T cell modulation in IRI, we analyzed the phenotype of CD4+ T cells in DC depleted mice after ischemia and reperfusion induction and we observed that the population of CD4$^+$ CD69+ (activated) T cells was increased, while no differences were observed in CD4$^+$ CD25+FOXP3+ T cells (Tregs), indicating that DC might be important for the regulation of T cell response in IRI.
Conclusions: We concluded that APC are involved in IRI and they modulate CD4+ T cells in order to control the injury.
Support: FAPESP, CNPq.

P0870

Assessment of CD8 positive NK cells and T cells subsets in pregnant women

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Purpose/Objective: The purpose of the study was to estimate the phenotype alterations in the peripheral blood NK and T lymphocyte subsets of women with unexplained pregnancy loss compared to healthy pregnant women.
Materials and methods: Fifteen women in the first trimester of pregnancy with a history of previous pregnancy loss (Group I), 11 non-pregnant women with a history of previous pregnancy loss (Group II), 12 women in the first trimester of pregnancy with no history of previous pregnancy loss (Group III), and 27 healthy fertile women with a history of successful pregnancies (controls) were included in the study. Five-color flow cytometric analysis was used to assess the percentage of CD3$^-$ CD56+ cells (NK), CD3$^+$ CD56+ T cells (NKT), CD8$^+$ CD11b$^+$ cells (CD8/CD11b) and their subsets: CD3$^-$ CD56$^+$ CD8$^+$ CD11b$^+$ NK cells (CD8+NK), CD3$^-$ CD56$^+$ CD8bright CD11b$^+$ NK cells (CD8brNK), CD3$^-$ CD56$^+$ CD8$^-$ CD11b$^+$ NK cells (CD8-NK), CD3$^+$ CD56$^+$ CD8$^+$ CD11b$^+$ NKT cells (CD8+NKT), CD3$^+$ CD56$^+$ CD8bright CD11b$^+$ NKT cells (CD8brNKT), CD3$^+$ CD56$^+$ CD8$^-$ CD11b$^+$ NKT cells (CD8-NKT), and CD3$^+$ CD56$^-$ CD8$^+$ CD11b$^+$ T cells (CD8+T).
Results: We found that the percentage of NK (8.9 ± 3.0) and CD8/CD11b (9.4 ± 4.7) in Group III was significantly lower compared to controls (12.6 ± 4.4; 13.4 ± 4.8 respectively). The percentage of these populations in Group I and Group II did not differ compared to controls. The percentage of NK in Group I (13.3 ± 4.9) was significantly higher, compared to Group III. The percentage of NKT in all the groups was nearly equal. A significantly lower percentage of CD8$^+$T was detected in Group III compared to controls (3.2 ± 2.4 versus 5.0 ± 1.8) and to Group II (3.2 ± 2.4 versus 6.6 ± 4.8). The percentage of CD8-NK and CD8-NKT cells was lower in Group III compared to Group I and Group II. The percentage of CD8+NK and CD8+NKT in all the groups was nearly equal. A significantly lower percentage of CD8brNK and CD8brNKT cells was detected in Group III compared to Group II.
Conclusions: Our results can suggest that, in addition to NK cells, T cells with a phenotype CD3$^+$ CD56$^-$ CD8$^+$ CD11b+ could be involved in the immune response during normal pregnancy. Further studies are needed to clarify the role of CD8$^+$ CD11b+ T cells, CD8+NK and CD8+NKT cell populations in complications of pregnancy.

P0871

Astrocytic FasL induces apoptosis of autoimmune T cells and is crucial for recovery from EAE

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Purpose/Objective: Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease characterized by accumulation of T cells in the CNS and its recovery requires the termination of inflammation and apoptosis of infiltrating T cells in the CNS. However, the T cell apoptosis inducing cell population still remains to be identified. To address the role of astrocytic FasL in the regulation of T cell apoptosis in EAE, astrocyte-specific FasL deficient mice were generated and investigated for EAE.
Materials and methods: Astrocyte-specific FasL knockout mice were generated by utilizing the Cre/loxP system under control of the human glial fibrillary acid protein (GFAP) promoter. EAE was induced by immunizing mice with MOG_{35-55} peptide and assessed daily by clinical score. Demyelination was detected by histology. Infiltrating leukocytes were isolated from spinal cord and characterized by flow cytometry. Expression of cytokines in spinal cord was analyzed by qRT-PCR. *In vitro* co-cultures of FasL$^+$ and FasL$^-$ astrocytes, respectively, with T cells were used to study the induction of T cell apoptosis as determined by annexin V and caspase-3 staining.
Results: FasL was efficiently and specifically deleted in astrocytes of GFAP-cre FasL$^{fl/fl}$ (KO) mice, while its expression was not affected in FasL$^{fl/fl}$ (control) mice. Compared with FasL$^{fl/fl}$ littermates, GFAP-cre FasL$^{fl/fl}$ mice developed significantly more severe EAE accompanied with widespread demyelination and more T cell infiltration. At day 22 post immunization, mRNA levels of IL-17, IFN-g, TNF and GM-CSF were also significantly elevated in the spinal cord of GFAP-cre FasL$^{fl/f}$

mice. Consistently, astrocytes isolated from GFAP-cre FasL$^{fl/fl}$ mice failed to induce T cell apoptosis *in vitro*.
Conclusions: FasL expression of astrocytes is required for the induction of T cell apoptosis and the elimination of infiltrating T cells in the CNS, which contributes to the recovery of EAE.

P0872
Calcitriol modulates the CD46 pathway by controlling its expression and function on T cells

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Purpose/Objective: The complement regulator CD46 is a costimulatory molecule for human T cells that induces a regulatory Tr1-like phenotype, characterized by large amounts of IL-10 secretion and low levels of IFNg. Secretion of IL-10 upon CD46 costimulation is largely impaired in T cells from patients with multiple sclerosis (MS). Vitamin D can exert a direct effect on T cells, and may be beneficial in several pathologies, including MS. Herein, we examined whether active vitamin D ($1,25(OH)_2D_3$ or calcitriol) could modulate the CD46 pathway and restore IL-10 production by CD46-costimulated T cells from patients with MS.
Materials and methods: CD4+ T cells were purified from the blood of 15 healthy donors and 11 patients with relapsing-remitting MS (7 IFNbeta treated, 4 untreated). We compared T cell responses to CD3/CD46 costimulation in presence or absence of calcitriol. We assessed the levels of expression of CD46, CD25 and CTLA-4 on activated T cells, and we measured proliferation and quantified IL-10 and IFNg production.
Results: In healthy T cells, calcitriol increased expression of CD25 and CTLA-4 expression on CD46-costimulated T cells, while it concomitantly decreased CD46 expression. Similar changes were observed in MS T cells except for CD25: while CD25 was normally induced by CD46 costimulation in MS T cells, addition of calcitriol consistently inhibited its induction. Despite the aberrant effect on CD25 expression, calcitriol increased the IL-10:IFNg ratio, characteristic of the CD46-induced Tr1 phenotype, in both T cells from healthy donors and patients with MS. Moreover, bystander CD4$^+$ T cells activated in presence of supernatants from CD46-costimulated T cells with calcitriol exhibited a lower proliferation rate.
Conclusions: We show that calcitriol affects CD46 expression and functions, and that it promotes anti-inflammatory responses mediated by CD46. Moreover, it might be beneficial for T cell responses in MS, although further studies should be performed to fully understand the effects of calcitriol on the CD46 pathway in T cells.

P0873
CCL17-producing dendritic cells at the crossroads of intestinal inflammation

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Purpose/Objective: The incidence of intestinal inflammations such as Crohn's disease and ulcerative colitis is constantly rising in Western countries. As current treatment options are not effective in all patients the need to find new drug targets is urgent. CCL17, a chemokine predominantly produced by dendritic cells (DCs) has been implicated to have proinflammatory roles in several atopic diseases as well as atherosclerosis. As central player of the innate and adaptive immune system, DCs play a fundamental role in the development of intestinal inflammation. Recent publications provided insights in the diversity of DC subsets involved in this process. In the work presented here, we assessed the role of CCL17 and CCL17-expressing DCs in particular, in murine experimental colitis.
Materials and methods: By challenging CCL17-deficient mice with chemically and immunologically induced colitis, the role of CCL17 was investigated. To characterize the different subsets of DCs cells within the secondary lymphatic tissues during colitis development multicolour FACS analysis and functional *ex vivo* assays were performed.
Results: CCL17-deficient mice displayed markedly reduced signs of inflammation after disease induction in two models of experimental colitis. Reduced disease development was associated with diminished levels of T_H1/T_H17 cells, as well as higher levels of Foxp3$^+$ regulatory T cells. Characterization of cell infiltrates into the lamina propria revealed that inhibition of colitis induction in CCL17-deficient mice was accompanied by increased numbers of CD103$^+$ DCs, as well as reduced expression of CCR7, crucial for recruitment to the mesenteric lymph nodes, on DCs within the colon. Further, the majority of CCL17-expressing DCs in the inflamed colon are found in the MHCII^{high}CD11b$^+$ CD103$^{+/-}$ CD8α^- intestinal DC subset, which was shown to have proinflammatory activity.
Conclusions: The work presented here clearly demonstrates a central role for CCL17 and CCL17-producing DCs in the development of intestinal inflammation. The in-depth characterization of these DCs will provide further essential insights in the processes shaping the intestinal immune response.

P0874
CD8 T cell – Dendritic Cell crosstalk up-regulates CD40L expression and IL-12p70 production

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Purpose/Objective: CD8 T cells have been shown to skew the immune response by stimulating dendritic cells (DC) to produce IL-12p70. The purpose of our study was to investigate the effect on CD8 T cell expression of CD40L.
Materials and methods: Ovalbumin (OVA)-specific T cell receptor transgenic mouse CD8 T cells (OT-I) were isolated by positive selection using anti-CD8 coated MACS beads and co-cultured splenic DC isolated using Optiprep and by positive selection with anti-CD11c coated MACS beads, and cultured in complete medium (RPMI 1640, 10% Fetal calf serum (FCS), antibiotics and non-essential amino acids and 0.5% 2 mercaptoethanol). CD40L expression was measured by Flow cytometry, IL-12p70 was determined by ELISA (R&D systems). CD8 T cells were activated with PMA (10 ng/ml) and Ionomycin (400 ng/ml) to induce CD44^{high} effector memory cells. CD11c+ splenic DCs were pulsed with 1 μg/ml of peptide (typically SIINFEKL) for 1 h before co-culture with pre-activated CD8 T cells at a ratio of 1 DC to 3 T cells. Altered SIINFEKL peptides (SAINFEKL, EIINFEKL, SIIRFEKL, SIINYEKL) were purchased from AnaSpec Inc (USA).
Results: Co-culture of effector memory CD8 T cells with DC up regulated CD8 T cell expression of CD40L that reached a maximum after 6 h. IL-12p70 levels in the culture reached 80% of its maximum value after 8 h. Altered peptide ligands SAINFEKL and SIINYEKL induced intermediate levels of CD40L while EINFEKYL and SIIRFEKL failed to induce CD40L on CD8 T cells. There was a corresponding reduction in IL-12p70 in the medium. Using CD8 T cells stimulated with anti-CD3 and CD28 addition of IL-12p70 enhanced CD40L expression on CD8s and addition of IL-12p70 neutralising antibody reduced CD40L expression.
Conclusions: CD8 T cell receptor dependent signals stimulate DCs to secrete IL-12p70 that up-regulates CD40L expression on CD8 T cells.

P0875

Characterization of swine skin and lung dendritic cells and their response to influenza virus

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Purpose/Objective: Swine is a natural host of influenza virus. Swine influenza strains can contaminate humans. Once infected, swine present identical symptoms as human, such as anorexia, pyrexia, cough, fever and nasal discharge. It has been recently shown in the mouse that dendritic cells (DC), and among them inflammatory TNF/iNOS-producing DC (Tip DC), are partly responsible both for the virus clearance and for the inflammatory pathology (Aldridge, PNAS 2009). Moreover, lung DC can be infected by influenza virus, although, interestingly, only the cross-priming CD103^{pos} DC subpopulation actually releases viral particles (Moltedo, PlosPathogen 2011). To establish if these DC/influenza interactions are idiosyncratic in the mouse model or can be generalized to natural hosts of influenza virus, we wanted:

1 To characterize the swine DC subpopulations network, first in the skin and then in the lung.

2 To describe their susceptibility to influenza virus infection and their capacity to produce new virions.

Materials and methods: Primary skin and lung cells were extracted and Facs-phenotyped (CadM1, CD206, CD209, CD14). Putative DC subpopulations were cell-sorted and the expression of genes specific for DC subpopulations (Flt3, ZBTB46, MAFB, BatF3, CSF1-R, XCR1, CX3CR1, CCR2, CLEC10A) were tested by q-PCR. Lung DC were infected with swine influenza virus and effective infection and virus production were tested by NP and NS1 immunofluorescence staining and by titration of the virus release.

Results: In a previous study, we described the different DC subpopulations present in pig skin (Marquet, PlosONE 2011). The deepening of this last study allowed us to revisit the classification of human skin DC subpopulations according to phenotypic and transcriptomic similarities between swine, human and mouse subsets, by reclassifying CD14^{pos} dermal DC as monocyte-derived DC and by identifying unambiguously the CD172a^{neg}/CadM1^{pos}/XCR1^{pos} cDC as equivalent to the murine CD103^{pos} cross-priming dermal DC.

We then identified the lung counterparts of these skin DC subpopulations and described their capacities to replicate influenza virus.

Conclusions: All together these data establish the swine as a model of choice for the study of normal and pathologic immune responses against influenza infection.

P0876

Circulating dendritic cells of multiple sclerosis patients are dysregulated and their frequency is correlated with MS-associated genetic risk factors

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Purpose/Objective: Dendritic cells (DC) are widely known as professional antigen-presenting cells and provide an important link to the adaptive immune system where they regulate the balance between immunity and tolerance. Alternations in the DC compartment can ultimately lead to the induction or perpetuation of autoimmune diseases such as multiple sclerosis (MS). This study aims to identify alterations in DC phenotype and functionality in MS. Moreover, the contribution of genetic risk factors to DC alterations was determined.

Materials and methods: An *ex vivo* analysis of myeloid (mDC) and plasmacytoid DC (pDC) was carried out on peripheral blood of MS patients (n = 104) and age- and gender-matched healthy controls (HC, n = 112). Frequencies and expression of costimulatory (CD80 and CD86) and migratory molecules (CD62L, CCR5 and CCR7) were investigated. Interleukin (IL)-12p70 and interferon (IFN)-α secretion was measured following Toll-like receptor (TLR) challenge. Study subjects were genotyped for HLA-DRB1*1501 and IL-7R α.

Results: A significant decrease of circulating pDC was found in peripheral blood of patients with chronic progressive MS (CPMS) compared to relapsing-remitting (RR) MS and HC. No differences in blood frequencies of mDC were found between different study groups. Both mDC and pDC of MS patients show shifts in the expression of CD86, CCR5 and CCR7 indicating that activation and migratory patterns of DC change during MS. Moreover, RRMS patients showed a reduced upregulation of CD86 on pDC and enhanced IL-12 production by mDC after TLR ligation, indicative of altered DC responsiveness. Treatment of MS is associated with a decrease of CD62L-positive mDC and pDC. HLA-DRB1*1501 carriers have reduced frequencies of circulating mDC as compared to non-HLA-DRB1*1501 carriers. Moreover, patients not carrying the protective IL-7Rα haplotype 2 have lower frequencies of pDC in the peripheral blood, indicating that genetic risk factors may impact the DC compartment of MS patients.

Conclusions: Our data indicate that circulating DC subsets undergo changes in phenotype and functionality during MS disease. This study further provides evidence that MS-associated genetic risk factors such as HLA-DRB1-1501 and absence of IL-7Rα haplotype 2 have an impact on the DC compartment and thereby may contribute to the induction and/or maintenance of autoimmune responses.

P0878

Contrasting responses of DC and NK cells to IFN-I contribute to host resistance to viral infection

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Purpose/Objective: Type I interferons (IFN-I) play a major role in immune defense against viral infections in mammals. Depending on the target cells, IFN-I either promote or inhibit cell proliferation and survival. How IFN-I mediate such opposite effects is not well understood. On dendritic cells (DC), IFN-I promote the up-regulation of major histocompatibility class I and co-stimulatory molecules, and the trans-presentation of IL-15. On natural killer (NK) cells, IFN-I promote entry into cell cycle, survival and cytotoxicity. However, the mechanism of action of IFN-I on NK cells, either directly by IFNAR triggering or indirectly through IL-15 presentation by DC, is still controversial.

Materials and methods: We combined the use of mutant mice, functional genomics, and flow cytometry analysis of transduction molecule phosphorylation to investigate how murine cytomegalovirus (MCMV) infection modulates the transcriptome and the antiviral activity of DC subsets and NK cells, in particular the role of IFN-I.

Results: We showed that splenic DC subsets undergo similar transcriptomic changes early after infection, with the induction of many IFN-I-stimulated genes, including genes involved in the recognition of viral infection, and inhibition of genes involved in proliferation. We showed that this occurs at least in part under cell-intrinsic instruction by IFN-I. NK cells induced genes involved in proliferation, which can be accounted for by stimulation through IL-15. We demonstrated that MCMV infection *in vivo* or IL-15 stimulation *in vitro* increase E2F expression and phosphorylation in NK cells, in a phosphatidylinositol 3-kinase dependent manner. Finally, we showed that the ability of DC, but not of NK

cells, to respond to IFN-I contributed to promote health over disease during MCMV infection.

Conclusions: In conclusion, we showed that DC and NK cells respond differently to IFN-I during a viral infection *in vivo*. DC are highly sensitive to IFN-I stimulation, due in part to their higher expression of STAT1/2. NK cells respond weakly to direct IFN-I stimulation but strongly to IFN-I-induced IL-15. This study demonstrates how contrasting responses of primary cell types to IFN-I can emerge as a result of combined quantitative differences in the signaling module downstream of IFNAR and qualitative differences in sensitivity to secondary cytokine mediators induced by IFN-I.

P0879

DCs expressing IDO controls the fungal loads, activation co-stimulatory molecules and T cells proliferation in PCM developed by B10.A and A/J mice

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Purpose/Objective: PURPOSE/OBJECTIVES: *Paracoccidioides brasiliensis* is a pathogenic fungus restricted to Latin America and its natural route of infection is the inhalation of fungal particles. In paracoccidioidomycosis (PCM), the regulatory mechanisms mediated by innate and cellular immunity are still unclear. Indoleamine, 23-dioxygenase (IDO) is an IFN-gamma induced enzyme which catalyzes the tryptophan metabolism along the kynurenine pathway. It is known that IDO can control host-pathogen interaction by inhibiting the proliferation of intracellular microorganisms due to tryptophan starvation and by its immunosuppressive effect on T cell immunity. The aim of our work was to investigate the influence of IDO on the behavior of dendritic cells (DCs) in the course of the infection of susceptible (B10.A) and resistant (A/J) mice clarifying some important aspects on the immunosuppression associated with the severe forms of PCM.

Materials and methods: We worked with B10.A and A/J mice using control and 1-methyl-DL-tryptophan (1MT, an IDO inhibitor)-treated mice. Control and 1MT-treated B10.A and A/J mice were infected i.t. with one million yeasts and analyzed at 96 h post infection. Dendritic cells of the lung were purified through magnetic beads and the parameters of influence of treatment with 1MT in IDO were analyzed such as mRNA expression; CFU counts; NO production; levels of kynurenine and cytokines; characterization of subpopulations of DCs present in the lungs; lymphocyte proliferation assay.

Results: In 96 h post infection of B10. A and A/J mice, 1MT was shown to decrease the frequency of DC cells expressing IDO and also the activation of co-stimulatory molecules CD40, MHC II and CD86. In both mouse strains IDO mRNA expression was augmented, driving tryptophan catabolism, kynurenine production and decreased fungal loads. On the other hand, 1MT restore proliferation of $TCD4^+$ and $TCD8^+$ cells in B10. A mice and $TCD8^+$ in A/J mice, showing a known effect on cells expressing IDO. In total lung cells (before magnetic beads) was observed the same effect of decreased co-stimulatory molecules, CD40, MHC II and CD86 in 1MT-treated mice.

Conclusions: CONCLUSION: DCs cells of resistant and susceptible mice use the enzyme IDO to control mechanisms of immunosuppression in infected mice, diminishing fungal burdens but also suppressing T cell proliferation.

P0880

DEF6, a Rho-GEF with a unique domain organisation involved in TCR mediated signal transduction aggregates via a coiled-coil domain to form cytoplasmic foci

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Purpose/Objective: Itk, a Tec family tyrosine kinase, regulates signalling molecules at the immune synapse including DEF6 (also known as SLAT or IBP), a Rho-GEF with a poorly characterised role in TCR mediated signal transduction (Gupta *et al. J Biol Chem* 2003, 278, 23541). Itk- and DEF6- deficient T cells both fail to activate ERK (Chen *et al. Immunity* 2008, *29*, 899; Schaeffer *et al. Science* 1999, *284*, 638), have decreased susceptibility to CD3-induced apoptosis (Schaeffer *et al. Science* 1999, *284*, 638), and exhibit aberrant IL-17 expression (Gomez-Rodriguez *et al. Immunity* 2009, *31*, 587). DEF6-deficient mice develop a systemic autoimmune disease similar to SLE (Fanzo *et al. J Clin Invest* 2006, *116*, 703), and rheumatoid arthritis (Biswas *et al. Immunol Rev* 2010, *233*, 79). Mice primed to develop experimental autoimmune encephalomyelitis lacking DEF6 showed resistance to its development (Canonigo-Balancio *et al. J Immunol* 2009, *183*, 7259). Our study aims to investigate the link between Itk and DEF6, and to explore the molecular mechanisms of DEF6 function.

Materials and methods: Phosphorylation assay.
Transient transfection with GFP tagged constructs.
Structural prediction.

Results: We have found that Itk phosphorylates the PH domain of DEF6 (submitted). This lays N-terminal to the DH-like domain (DHL), a feature unique to DEF6 and its B cell homolog, SWAP70. Transient transfection of a DEF6 mutant mimicking Itk phosphorylation forms cytoplasmic foci in Cos7 cells that co-localize with DCP1, a marker of P-bodies; structures containing machinery to degrade RNA (Kedersha *et al. Methods Enzymol* 2007, *431*, 61). The DHL domain alone can form foci that do not co-localize with DCP1. Whilst the DHL domain exhibits GEF activity, structural prediction suggests it does not form the usual Rho-GEF DH domain structure. Additionally, the amino acid composition of the DHL domain is characteristic of a group of prion proteins (Fiumara *et al. Cell* 2010, *143*, 1121) and some P-body proteins. In DEF6 this region is likely to form coiled-coil rather than beta-sheet based aggregation to drive the formation of the foci.

Conclusions: Our data suggest that the atypical domain structure of DEF6 is responsible for maintaining a meta-stable conformation, easily changed by phosphorylation to promote GEF activity and/or cytoplasmic granule formation potentially involved in the regulation of translation.

P0881

Dendritic cell mediated immune reactions to mycobacterial antigens in young healthy BCG vaccinated individuals

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Purpose/Objective: Dendritic cells (DC) are indispensible in initiation of adaptive responses to infective agents. The aim of study was to analyze the relationship between mycobacterial antigen induced responses of monocyte derived dendritic cells (DC) manifested on the level of surface receptors and cytokine production and capacity of mycobacterial antigen pulsed DC to stimulate autologous CD4, CD8 and CD56 cells. The study was performed in healthy subjects

(17–30 years old) who were given 2 or 3 doses of BCG vaccine at their childhood.
Materials and methods: Blood CD14+ monocytes separated with immunomagnetic method, cultured with IL-4 and GM-CSF gave DC. The receptors on DC stimulated with PPD or BCG bacilli and the phenotype and intracellular IFN-gamma in CD14-cell fractions responding to mycobacteria-pulsed DC were estimated by flow cytometry. ELISA was used for released cytokine detection.
Results: An individual variation was noticed in the responses of DC to PPD and whole BCG bacilli manifested on the level of co-receptors CD86, CD80, CD40, HLA-DR and DC-SIGN as well as IL-10 and IL-23 releasing. However, PPD and BCG caused a significant decrease in DC-SIGN on DC from almost all volunteers. As opposed to PPD, BCG enhanced a CD86 density on and IL-10 releasing by DC in the majority of donors. A high frequency of IL-10 production characterized DC from tuberculin-negative volunteers. An antigen presentation function of PPD or BCG pulsed DC towards autologous CD4+, CD8+ and CD56+ cells was estimated as an intracellular IFN-gamma. A great individual variation in the profile of responding T and NK cells was observed. There was a positive correlation between HLA-DR expression and IFN-gamma response of CD56+ cells stimulated with PPD-pulsed DC and a negative correlation between HLA-DR density and intracellular IFN-gamma in total lymphocytes.
Conclusions: The complexity of DC mediated immune reactions to mycobacteria requires regarding in a designation of better vaccines than BCG. Supported by the MS grant N N401 015236

P0882
Dendritic cells orchestrate innate immunity against bacterial kidney infection

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Purpose/Objective: Urinary tract infections, such as pyelonephritis (PN), affect a large proportion of the population worldwide and are mostly caused by the uropathogenic gram-negative bacteria *Escherichia coli* (UPEC). Dendritic cells (DCs) form an abundant network in the kidney tubulointerstitium. Their role in bacterial pyelonephritis is unknown. Here we studied that role in a murine pyelonephritis (PN) model induced by transurethral instillation of UPEC.
Materials and methods: Instillation of UPEC twice at a 3 h interval increased infection rates from 16% after a single instillation to 84%. To investigate the role of DCs in early PN we used CD11c transgenic deleter mice to deplete DCs with 8 ng/gbw diphtheria toxin. After kidney digestion renal phagocytes were intracellularly stained for CXCL2 and TNFa and were analyzed by flow cytometry.
Results: Already 3 h after the second instillation, resident kidney DCs produced most of the intrarenal CXCL2 and TNFa. These cells recruited and activated neutrophilic granulocytes, which are critical in the defense against PN. When we depleted DCs using CD11c. DTR mice during the first bacterial instillation, neutrophil recruitment as well as bacterial clearance was markedly delayed. However, DC depletion also caused infection-independent granulocyte release from the bone marrow commencing after 24 h. The resulting neutrophilia paradoxically improved bacterial clearance when DCs were depleted 1 day before infection. This side effect was also seen in CD11c. DOG mice, another transgenic line allowing conditional DC depletion. Here we introduce a new transgenic line, CD11c. LuciDTR mice, which is unaffected by such early neutrophilia. However, both CD11c. LuciDTR mice and CD11c. DTR mice, but not CD11c. DOG mice, showed neutrophilia after 72 h, which probably resulted from increased granulopoiesis.
Conclusions: All three lines feature time-windows, during which neutrophilia is negligible. Studies within these time windows allowed us to demonstrate that the tubulointerstitial DC network serves an innate immune sentinel function against bacterial pyelonephritis, by rapidly recruiting neutrophils into the infected kidney.

P0884
E2-2-mediated down-regulation of plasmacytoid dendritic cell function limits type I interferon, T cell responses and virus control in early life

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Purpose/Objective: Cytopathic viruses causing acute infections in immunologically mature hosts often follow a prolonged course in early life, characterized by high viral titers maintained for several weeks. Accordingly, lymphocytic choriomeningitis virus (LCMV, WE strain) runs an acute course in adults but a protracted course in infant BALB/c mice, in which LCMV-specific T cells fail to expand and control infection. To identify whether these defects were due to a dysfunction of innate immunity in infant mice, we analyzed early events of viral infection.
Materials and methods: Type I IFN production was assessed early after LCMV infection of infant and adult mice by ELISA and real-time PCR. We compared viral titers (by plaque assay) and T cell responses (by FACS) of LCMV-infected infant/adult wild-type and infant/adult IFNAR $^{-/-}$ mice. Activation and function of plasmacytoid and conventional dendritic cells were analyzed by FACS and real-time PCR. The effect of supplementing recombinant IFN-alpha on viral control and T cell responses was investigated in infant mice.
Results: Our data showed an insufficient immediate-early IFN-alpha production in infant mice, which fails to support early viral control and the expansion of LCMV-specific T cells: disrupting IFNAR signaling in adult mice mimicked a protracted LCMV infection. Plasmacytoid dendritic cells (pDCs) which are the main source of type I IFNs in LCMV infection failed to acquire an activated phenotype in infant mice and displayed defective function *in vivo* upon LCMV infection reflected by the low expression of the central pDC transcription factor E2-2 and related genes (TLR7/9, IRF-7 and IRF-8), MyD88 and NF-KappaB. In contrast, the *in vitro* function of early life pDCs was normal suggesting an *in vivo* negative regulation of infant pDCs. Direct evidence of the contribution of type I IFNs for early life infection control was demonstrated by given exogenous IFN-alpha which restored virus control and CTL functionality in infant mice.
Conclusions: In this study, we identify an age-specific down-regulation of multiple factors which are critical for the activation and function of pDCs and demonstrate that it is orchestrated by the E2-2 pDC regulator. We show that this down-regulation prevents the immediate-early burst of type I IFN and the early viral control and permanently impairs T cell responses. This defect was overcome by giving exogenous IFN-alpha which restored virus control and T cell responses in infant mice. The results suggest that early life pDC responses are tightly regulated *in vivo*, possibly to avoid potentially harmful inflammatory or autoimmune reactions.

P0885
Effect of Th17/Th1/Treg balance and microRNA expression in *A. actinomyctemcomitans* – accelerated atherosclerosis

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Purpose/Objective: Recent studies have shown that there is an association between periodontal disease and cardiovascular disease. We

previously reported that the TLR signaling pathway plays an important role in *Aggregatibacter actinomyctemcomitans (A. a.)* mediated atherosclerosis in apolipoprotein E-deficient spontaneously hyperlipidemic ($Apoe^{shl}$) mice. However, it is not known whether the causal or primary immune mechanism is involved in periodontopathic bacteria-associated atherosclerosis. Atherosclerosis is a chronic inflammatory disease regulated by T lymphocyte subsets. In this study, we investigated whether the functional imbalance between Th17, Th1, and regulatory T (Treg) cells, and Th17-related microRNA expression existed in *A. a.*-challenged $Apoe^{shl}$ mice.

Materials and methods: The mice were intravenously treated with live *A. a.* HK1651 or vehicles. Histomorphometric features of atheromatous lesions, serum IFN-gamma, IL-17 and IL-10 levels, and gene expression of Th17-related molecules and microRNA (miR) were examined.

Results: We observed that *A. a.* challenge induced a Th17/Th1 shift in $Apoe^{shl}$ mice. *A. a.*-challenged splenic Th17 cells greatly increased during early stages of bacteria-challenge, compared to Th1 cell increase accompanied with Th17 cell reduction during later stage of challenge. In contrast, splenic Treg cell-changes in the *A. a.*-challenged group were almost similar with the control group. Similarly, differentiation factors (TGF-βplus IL-6, TL-17RA and IL-21), growth and stabilization factor (IL-23), and transcription factor (STAT3) involved in the development of Th17 cells, as well as Th1-related IFN-γ were also detected in *A. a.*-challenged mice. Furthermore, we found that miR-326 expression was upregulated in the hearts of *A. a.* − challenged mice.

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Conclusions: These results suggest that Th17/Th1/Treg imbalance and Th17 cell-associated miR-326 expression exists during early atherosclerosis in *A. a.*-challenged $Apoe^{shl}$ mice, suggesting a potential role of Th17/Th1/Treg imbalance in the progression of *A. a.*- accelerated atherosclerosis.

P0888
Enhanced CD8 T cell priming in DAP12 and FcR-gamma deficient mice

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Purpose/Objective: The disruption of the immunoreceptor tyrosine-based activation motif (ITAM) signaling in mice with combined deficiency in DAP12 and FcR-gamma (DF mice) was shown to have either positive or negative effects on dendritic cell function. However, the exact role of dendritic cell ITAM (DC ITAM) signaling in CD8 T cell priming was not explored so far.

Materials and methods: To determine the requirement of DC ITAMs in CD8 T cell priming, we performed a series of immunization experiments with MHC class I restricted peptide ($OVA_{257-264}$) to evaluate CD8 T cell priming followed by analysis of dendritic cell subsets and evaluation of IFN type I and cytokine responses.

Results: We found sharply increased CD8 T cell priming in DF mice, as indicated by increased frequency of IFN-gamma producing cells after re-call response with $OVA_{257-264}$ peptide (SIINFEKL) *in vitro*. Given that many DC subsets contribute to modulation of T cell responses *in vivo* we hypothesized that the difference in CD8 T cell priming could be related to activation of specific DC subsets resulting in polarization of the T cell response. In this line of investigation we found an increased number of monocyte-derived- and plasmacytoid dendritic cells in the draining lymph nodes of DF mice after immunization with SIINFEKL. We tested if there was a cell autonomous defect due to ITAM signaling deficiency, and noted no difference in the ability of sorted CD8α DCs from DF and WT mice to prime exogenous CD8 T cells *ex vivo*. Subsequently, given that IL-12 significantly contributes to increased IFN-gamma production, we noted that monocyte-derived DCs (moDC), from bone-marrow of DF mice, expanded after GM-CSF culture produced increased amounts of IL-12, along with up-regulation of transcription factor IRF-8. Consistent with this scenario the treatment of DF mice with IFNAR1-specific MAR1-5A3 mAb followed by the immunization with SIINFEKL, showed dramatically decreased IFN-gamma production.

Conclusions: Collectively, we showed that DC ITAMs negatively regulate IFN type I responses and IL-12 production controlling CD8 T cell priming.

P0889
Enteropathogenic *Escherichia coli* engages in temporal regulation of the inflammasome by interaction with NLRC4 and NALP3

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Purpose/Objective: Enteropathogenic *Escherichia coli (EPEC)* is a leading cause of acute and chronic diarrhoea in developing nations, principally affecting children <2 years of age. The interaction of EPEC with the gastrointestinal (GI) mucosal immune system remains largely uncharacterised. A recent study by Lebeis *et al.* 2011 indicated a critical role for IL-1 receptor signalling in Citrobacter rodentium (murine model for EPEC) mediated disease pathogenesis. In the present study we investigate the signalling events that regulate IL-1β production in murine dendritic cells in response to EPEC infection. Better understanding of this complex pathway may highlight novel therapeutic targets for infection control and highlight strategies for vaccine development for this major enteropathogen.

Materials and methods: Bone marrow derived dendritic cells (BMDC) from inflammasome knockout mice $NLRC4^{-/-}$, $NALP3^{-/-}$, $ASC^{-/-}$, $CASPASE^{-/-}$ were isolated and infected with EPEC Wild Type (WT) strain (E69) and isogenic mutants deficient in structural and effector protein of T3SS (Type III secretion system) and flagella to determine inflammasome activation. Inflammasome activation was assessed by gene and protein expression, flow cytometry and florescent microscopy.

Results: WT strain induced marked increase in bioactive IL-1β in a caspase dependant manner in $NLRC4^{-/-}$ BMDC. This effect was dependant on the presence of T3SS component in EPEC. In contrast to published work by Miao *et al.* (2010), we found a minimal role of flagella mediated IL-1β as ΔFliC elicited IL-1β to similar levels as WT in $IPAF^{-/-}$ DC. ASC speck formation an essential adaptor required for regulating mature caspase by the inflammasome showed a significant decrease in both $NLRC4^{-/-}$ and $NLRP3^{-/-}$ suggesting temporal regulation of both in ASC mediated caspase activation.

Conclusions: An intact T3SS was found to be necessary for optimal IL-1β release. Our experiments indicate potential role of ASC in EPEC-mediated IL-1β release especially in the presence of the inflammasome NLRC4. Our study is the first to show the effect of both NLR (Nod-like Receptors) in a time dependant manner.

P0891

Expression of HLA-B27 causes loss of migratory dendritic cells in a rat model of spondyloarthritis

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Purpose/Objective: The Spondyloarthritides (SpAs) are a group of related inflammatory disorders that share common clinical features and a genetic predisposing factor, the MHC class I gene HLA-B27. Rats transgenic for human HLA-B27 and b2-microglobulin (B27-TG rats) spontaneously develop colitis and peripheral inflammation, and thus provide a model of SpA. Because inflammation in the animals requires $CD4^+$ T lymphocytes and involves intestinal pathology, we hypothesized that the dendritic cells (DCs) that migrate from the intestine and control $CD4^+$ T cell differentiation would be aberrant in B27-TG animals.

Materials and methods: Migrating intestinal lymph DCs were collected by thoracic duct cannulation from B27-TG and control (HLA-B7 transgenic or non-transgenic) rats. The phenotypes of these DCs, and of mesenteric lymph node DCs, were assessed by flow cytometry. Also, the ability of DCs to differentiate from bone marrow precursors *in vitro* was assessed.

Results: Strikingly, B27-TG animals lack one of the three subsets of migrating lymph DCs, the $MHCII^{hi}$ $CD103^+$ $CD172a^{lo}$ cells, in the pseudo-afferent lymph. This '$CD172a^{lo}$' DC subset has been implicated in the induction and maintenance of intestinal tolerance, and thus lack of this subset could lead to breakdown in tolerance and to systemic disease. In addition, the remaining B27-TG L-DCs express more CD25, indicating an increase in their activation. The $CD172a^{lo}$ subset was also proportionally reduced in the B27-TG mesenteric lymph nodes. Furthermore, *in vitro* culture of DCs from bone marrow precursors with Flt3L revealed a reduced survival of B27-TG DCs, suggesting a systemic defect in B27-TG DC differentiation. In spite of the reduced viability of B27-TG BMDCs, the DCs that were generated *in vitro* induced enhanced IL-17 production from naïve $CD4^+$ T cells.

Conclusions: We describe two different mechanisms by which HLA-B27 may contribute to inflammatory disease: increased apoptotic death of B27-TG DCs that normally function to maintain immunological tolerance; and enhanced IL-17 production from $CD4^+$ T cells stimulated by the surviving B27-TG BMDCs.

P0892

Granzyme B produced by human plasmacytoid dendritic cells is regulated by anti-viral vaccines

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Purpose/Objective: Plasmacytoid dendritic cells (pDC) are decisive modulators of adaptive T cell responses. Recently we showed that human pDC can produce large amounts of granzyme B (GrB) and that such GrB^+ pDC potently suppress T cell proliferation in a GrB-dependent, perforin-independent manner, reminiscent of regulatory T cells. The relevance of this phenomenon for anti-viral immune responses is not clear.

Materials and methods: The study was performed with pDC isolated from healthy volunteers treated with commercially available anti-viral vaccines *in vitro*. GrB and IFN-alpha production was measured using glow cytometry and ELISA, induction of T cell proliferation was analyzed in pDC- T cell cocultures. In a subset of experiments, pDC were derived from healthy subjects before and after vaccination with tick-borne encephalitis (TBEV) vaccine.

Results: We demonstrate that commonly used anti-viral vaccines against infections including TBEV, poliomyelitis and measles potently suppress pDC-derived GrB production and secretion, thereby paving the way for a more efficient capacity of vaccine-treated pDC to induce T cell proliferation. PDC derived from recently TBEV-vaccinated donors produce less GrB after than before vaccination. TBEV, the most potent enhancer of pDC-induced T cell proliferation, inhibits GrB transfer from IL-3-stimulated pDC to T cells and leads to marked IFN-alpha production in pDC.

Conclusions: Alteration of GrB-secreting pDC activity may be a mechanism by which anti-viral immune responses are regulated. Our results provide novel insights into pDC-T cell interactions and may contribute to an advancement of prophylactic and therapeutic vaccinations.

P0893

HLA class II tetramer detection of a-enolase specific T cells in rheumatoid arthritis

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Purpose/Objective: Antibodies against different citrullinated proteins are highly specific for RA and α-enolase is one candidate autoantigen towards which antibodies are found. These autoantibodies are strongly associated with the RA risk factor HLA-DRB1*0401. Here, we have studied a set of novel HLA-DRB1*0401 restricted T cell epitopes from α-enolase and constructed and utilized HLA class II tetramers to determine the frequency of α-enolase-specific T cells in blood and synovial fluid of DRB1*0401 RA patients.

Materials and methods: HLA-binding assays were performed for putative T cell epitopes. After verifying positive HLA-DRB1*0401 binding of the candidate peptides, PBMC were cultured with peptides to determine intracellular production of IFN-γ, IL-17A and TNF. Furthermore, we constructed HLA class II tetramers loaded with the peptides to screen mononuclear cells from peripheral blood (PBMC) and synovial fluid of CCP+ HLA-DRB1*0401 RA patients for α-enolase specific T cells. T cells recognizing the α-enolase peptides were visualized using direct *ex vivo* multi-color flow cytometry analysis including phenotypic surface markers.

Results: Novel T cell epitopes in α-enolase were identified; one where only the citrullinated form bound HLA-DRB1*0401 and another where both the native and citrullinated version formed pMHC complexes (with similar affinity). Upon *in vitro* culturing of PBMC with each of the three T cell epitopes, intracellular production of proinflammatory cytokines was apparent and could be prevented by addition of an HLA-DR blocking antibody. Next, HLA class II tetramers were constructed and utilized for the detection of α-enolase specific T cells; in peripheral blood 7/12 RA patients displayed α-enolase specific cells while 9/12 synovial fluids were positive. The *ex vivo* frequency of influenza HA-specific T cells were similar in both peripheral blood and synovial fluid, while the frequencies of α-enolase positive T cells varied greatly between individuals and compartments. Still, the highest frequency of α-enolase specific T cells was found in synovial fluid. Overall, most tetramer positive cells (both HA and in particular α-enolase) where of memory CD45RO phenotype and did not express CD25.

Conclusions: We have identified novel α-enolase T cell epitopes that bind HLA-DRB1*0401. T cells specific for these pMHC's were commonly found in RA patients and produced proinflammtory

cytokines. These α-enolase specific T cells may participate in disease driving immune responses and potentially serve as candidates to immune modulatory strategies.

P0894
HLA-B and HLA-DRB1 associations with henoch-schönlein purpura

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Purpose/Objective: Henoch-Schönlein purpura (HSP) is the most common vasculitis in children with an estimated incidence of 20 in 100 000 children. The pathogenesis of the disease still remains unknown and its genetic susceptibility has been mainly associated to the HLA system. Increased prevalence of some HLA-DRB1 and HLA-B alleles has been previously described and also associations with nephritis and its severity. The aim of this study was to investigate HLA-B and DRB1 genotype frequencies in HSP patients of our area (Gran Canaria, Spain).
Materials and methods: Twenty-four children diagnosed with HSP (9 female, 15 male; Age range 4−16 years) and 121 controls (Blood donors) were enrolled in the study. HLA-B and DRB1 DNA typing was performed with PCR-SSP commercial kits (Innotrain). Differences in frequencies were assessed by the chi-square test.
Results: HLA-B51 was found to be significantly increased in patients compared to controls (14.6% versus 5.8%, $P = 0.04$). Previously described associations from some other areas in Europe such as B35 were not found in our area (8.3% versus 9.9%) HLA-DRB1*04 was increased in patients (25% versus 12.1%, $P = 0.01$) and DRB1*13 was decreased (10.4% versus 23.7%, $P = 0.04$). We did not find differences regarding the severity of the nephritis among the patients.
Conclusions: In this preliminary study HLA-B51 seems to be a factor for susceptibility to the disease as previously described in a cohort from Madrid (Spain). Regarding HLA-class II, HLA-DRB1*04 is also increased in patients while HLA-DRB1*13 was found to be negatively associated with HSP. These results differ from studies carried out in Italy, Northwest Spain and Turkey.

P0896
IgG-opsonization of HIV attenuates the CTL-stimulatory capacity of dendritic cells

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Purpose/Objective: Control of HIV is suggested to depend on potent effector functions of the virus-specific $CD8^+$ T cell response. Antigen opsonization can modulate the capture of antigen, its presentation and the priming of specific $CD8^+$ T cell responses. We have previously shown, that opsonization of retroviruses acts as endogenous adjuvant for DC-mediated induction of specific CTLs. However, in some HIV-positive individuals, high levels of antibodies and low levels of complement fragments coat the HIV surface.
Materials and methods: Thus, we analyzed the impact of IgG-opsonization on the antigen-presenting capacity of dendritic cells (DC) by $CD8^+$ T cell proliferation assays following repeated prime-boosting with loaded DC, by measuring the antiviral activity against HIV-infected autologous $CD4^+$ T cells, and by IFN-γ secretion from HIV-specific CTL clones.
Results: We found, that IgG deposition on the viral surface was associated with a loss of the CTL-stimulatory capacity by DC as represented by reduced proliferation, a weak antiviral activity and significantly lower activation of HIV-specific CTL clones compared to HIV covered with high amounts of C and low IgG concentrations.
Conclusions: Our results illustrate the impact of the opsonization pattern of HIV in DC-induced expansion and differentiation of specific CTLs.

P0897
Immune complexes enhance uptake of blood coagulation factor VIII by antigen presenting cells

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Purpose/Objective: The X-chromosome-linked bleeding disorder hemophilia A is caused by the absence or dysfunction of blood coagulation factor VIII (FVIII) which can be corrected by regular intravenous administration of FVIII. Development of antibodies directed against FVIII (referred to as FVIII inhibitors) is common complication in hemophilia care. Immune tolerance induction (ITI) comprising frequent administration of high doses of FVIII is the treatment of choice for hemophilia A patients with inhibitors. Thus far, the molecular mechanisms contributing to tolerance induction have not been defined. Here we sought to study the role of immune complex (IC) formation in endocytosis and functional presentation of FVIII by antigen presenting cells.
Materials and methods: Bone marrow-derived dendritic cells (DC) were used as model antigen-presenting cells, as DCs are known for their ability to take up and process immune complexes via FcγR. Purified recombinant FVIII was labeled using the Microscale Alexa Fluor 488 protein-labeling kit. For T-cell proliferation assays, $FVIII^{-/-}$ mice were injected intravenously 5× weekly with 1 μg of recombinant FVIII. Subsequently, spleens collected after weekly injections were processed into single-cell suspensions and used as a source of FVIII-specific T cells.
Results: Bone marrow-derived murine DCs were able to efficiently take up FVIII pre-complexed with anti-FVIII antibodies (FVIII-IC) in a dose-dependent manner. Endocytosis of FVIII-IC was 5- to 10-fold more efficient when compared to equimolar concentrations of soluble FVIII. Uptake of FVIII-IC, but not FVIII alone, could be inhibited with 2.4G2 antibody indicating functional involvement of FcγRI/III in this process. Moreover, antibodies used for IC formation were analysed based on isotype IgG2a antibodies were most efficient in mediating IC endocytosis. Experiments performed with FVIII-specific T cells showed that in the low concentration range (<1 nM), FVIII-IC induced stronger T cell proliferation when compared to soluble FVIII.
Conclusions: Collectively, these data provide further insight and understanding of modulation of anti-FVIII immune response in hemophilia A patients with pre-existing FVIII inhibitors.

P0900
Immunosenescence and dendritic cells: role of TLR2 and TLR4 in phagocytosis and cytokines production against Candida albicans

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Purpose/Objective: Purpose.

Dendritic cells (DC) are the most potent antigen presenters, and play a key role in the induction of both innate and adaptive immunity.

Aging is associated withprogressive decline in immune function, resulting in an increasing susceptibility to infection, taking into account the specific changes in T cells, macrophages, neutrophils and DC, usually referred to as immunosenescence. Among microorganisms, the fungus *Candida albicans* (*C. albicans*) is an important pathogen for the development of invasive infections, especially in immunocompromised individuals and elderly persons. In addition, functional alterations in DC with aging, including the role of *C. albicans* infection are poorly understood.

Objectives.

To study the phagocytic capacity and aspects of immunomodulatory function of mature DC against *C. albicans* in aged patients, through the production of TNF-α, IL-6, IL-23 and IL-10. The involvement of TLR2 and TLR4 receptors was also evaluated.

Materials and methods: Peripheral blood monocytes were isolated from healthyelderly donors ($n = 18$) and young donors ($n = 20$), and were cultured for 7 days to differentiate into DC under the influence of IL-4 and GM-CSF. Participation of receptors type TLR2 and TLR4 was assessed *in vitro* by using specific blockingantibodies. Cytokine production was measured by ELISA test and phagocytosis assays were realized for 1 h and analyzed by confocal laser scanning microscopy. The data was statistically analyzed and $P < 0.05$ was considered significant.

Results: TNF-α, IL-10 and IL-23 production by DC was higher in young than aged subjects, spontaneously or after *C. albicans* challenge. The elderly presented more DC with internalized *C. albicans* than young individuals (89.7% and 63.3%, respectively). The TLR2 or TLR4 blocking did not affect cytokine production or phagocytosis by DC.

Conclusions: Together, these data suggest an impairment of cytokine production by DC from elderly in comparison with young subjects. Moreover, the elderly presented more DC with internalized fungus than young subjects, which would mean failure in time to kill the phagocytosed microorganism. Probably, receptors other than TLR2 and TLR4 are involved in signaling to elicit phagocytosis or cytokine production by DC.

P0901
Impact of cross-linking CD161 on human T cells

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Purpose/Objective: CD161 is a C type Lectin expressed on NK cells and subsets of T cells. CD161 is one of the markers used to define Th17 cells. Human mucosal associated invariant T cells (MAIT) have been shown to express high levels of CD161 ($CD161^{++}$). In chronic viral hepatitis C there are tissue infiltrating T cell populations that express high levels of CD161. The CD161 protein is known to be a marker for these important cell subsets, however we do not yet understand the impact of CD161 triggering on T cell function. Understanding the functional impact that CD161 triggering has on the function or regulation of MAIT, Th17, Tc17 and other T cell subsets in human inflammatory diseases, are potentially of relevance to their role *in vivo* and therapeutic manipulation.

Materials and methods: To study the effects of cross-linking CD161 lymphocytes, (PBMCs) were isolated from healthy volunteers, stimulated and the CD161 on cells was cross-linked. Intracellular cytokine staining (ICS) and fluorescence-activated cell sorting (FACS) were used to look at the effect of cross-linking CD161 on production of IFN-g, other cytokines and other cell surface markers. We repeated these experiments using the physiologic ligand, LLT-1 (CLEC2D), expressed as a recombinant protein or on transfected cells.

Results: We stimulated PBMCs with multiple different stimuli, including PMA/I, aCD3 and aCD28, *E. coli* stimulated THP1 cells, IL12, and IL-18. We were unable to show an effect on the production of IFNy by $CD161^{+}$ T cells using antibody cross-linking or stimulation with the physiologic ligand under any condition. However, we were able to consistently show a clear dose dependent down regulation of CD161 surface protein on cells with high levels of CD161 expression ($CD161^{++}$).

Conclusions: Cross-linking CD161 via a range of stimuli induces a dose-dependent downregulation of CD161 on $CD161^{++}$ cells without affecting cytokine production. Further investigation is needed to define the key functional impact of CD161 ligation/downregulation on these critical cell subsets.

P0902
Impact of nitration on the immunogenicity of Bet v 1.0101

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Purpose/Objective: Nitration of Bet v 1.0101 (Bet) can occur exogenously and endogenously, and might be one explanation for the higher prevalence of allergy in industrialised countries. Previously, nitration was shown to influence processing of allergens by dendritic cells (DCs). The present study investigates effects of nitration on Bet-specific IgE reactivity, Bet-dependend DC-activation and proliferation of Bet-specific T cell lines.

Materials and methods: IgE reactivity against freshly nitrated Bet and mock-Bet was tested in 100 patient sera by ELISA. In a second setup, monocyte derived DCs were stimulated with Bet, mock-Bet and nitro-Bet, and the secreted cytokines were analyzed, as well as the surface markers. Additionally, specific T cells were isolated from allergic patients and the proliferation upon stimulation with the unmodified and the nitrated allergen was measured. To follow the biophysical changes occurring upon nitration gel electrophoresis, amino acid analysis and size exclusion chromatography were performed.

Results: No increased IgE response towards nitro-Bet versus mock-Bet could be found in the sera of allergic patients. Analysis of the cytokines secreted by monocyte derived DCs reveals high concentrations of the Th1 promoting cytokines IL-12, IL-6 and TNF-α upon stimulation with Bet, which is probably due to lipopolysaccharide bound to the allergen. Notably, this Th1 priming milieu is absent in the case of nitrated Bet. Furthermore, nitrated Bet induces a much stronger proliferation of Bet specific T cells, compared to the proliferation of T cells after stimulation with Bet or mock-Bet. The main biophysical change due to nitration was the induction of SDS resistant allergen oligomerisation of nitro-Bet.

Conclusions: The data obtained in this study demonstrate that nitration of Bet v 1.0101 results in a higher immune response, possibly due to consequent oligomerisation. These findings strongly suggest that nitration plays a role in the higher prevalence of allergy.

P0903

Increased immunogenicity of proteins modified by hypochlorous acid, may be caused by their enhanced uptake via endocytic receptors expressed on antigen presenting cells

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Purpose/Objective: During respiratory burst, neutrophils, eosinophils and monocytes produce hypochlorous acid (HOCl) which, apart from contributing to killing of pathogens, may also oxidize different proteins at sites of inflammation. It was previously reported that *in vitro* presentation of HOCl-modified ovalbumin (OVA-Cl) to ovalbumin (OVA)-specific lymphocytes by dendritic cells (DCs) was more effective than that of native OVA. Moreover, mice immunized with OVA-Cl produced higher amounts of OVA-specific antibodies that mice immunized by native OVA. We reported previously that OVA-Cl is subjected to strongly enhanced uptake by macrophages and DCs. This increased uptake and intracellular processing may be the major mechanism of increased immunogenicity of OVA-Cl as OVA-Cl neither stimulated cytokine release nor modulated expression of MHC class II and co-stimulatory molecules on the surface of DCs.
Materials and methods: We examined receptors involved in OVA-Cl uptake with the use of fluorescently labeled ligands, receptor-blocking antibodies, receptor-deficient cells and recombinant receptors.
Results: We found that two types of receptors: one blocked by dextrans sulfate (DS), but not by mannan, and the second one, blocked by both DS and mannan, contributed almost equally to OVA-Cl uptake by DCs. Results of further experiments revealed that the mannose receptor (CD206) accounted at least partially for the portion of OVA-Cl uptake which is inhibited by both mannan and DS, whereas different scavenger receptors, including scavenger receptor A (SR-A), LOX-1 and SREC-I, may be responsible for the part of the uptake which is inhibited by DS only. Finely, we have demonstrated that upon chlorination other unrelated proteins and glycoproteins gain the ability to bind to the same receptors as OVA-Cl.
Conclusions: Our results indicate that increased immunogenicity of chlorinated proteins is caused by their increased uptake by DCs through several endocytic receptors, including the mannose receptor, SR-A, LOX-1 and SREC-I. We postulate that oxidation by HOCl may be a universal mechanism of increasing immunogenicity of proteins which in their native forms are not recognized by endocytic receptors present on the surface of antigen presenting cells.

P0904

Increased Th22 cells in patients with Beçhet's disease

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Purpose/Objective: Beçhet's disease (BD) is a systemic inflammatory disease with recurrent inflammatory mucosal ulcerations, ocular, vascular, gastrointestinal, musculoskeletal and central nervous system involvement. Although innate immune system plays the major role in the pathogenesis, previous data has shown that some T helper (Th)1 associated cytokines have role in the inflammation of BD. The $CD4^+$ Th cells can be differentiated into Th1, Th2, Th17, Th22 and regulatory T (Treg) cells and secrete different cytokines to regulate immune system. In this study cytokine secretion of Th cells in BD were investigated.
Materials and methods: Twenty-six patients with BD that have not received any immunosuppressive treatment ($n = 26$, mean age = 37.2 ± 9.7) with mucocutaneous involvement and healthy subjects ($n = 12$, mean age = 32.5 ± 8) were enrolled in this study. Lymphocyte subpopulations in peripheral blood samples and intracellular cytokine secretion including IL-5, IL-10, IL-17, IL-22 and IFN-γ of $CD4^+$ T and $FOXP3^+$ Treg cells were determined by flow cytometry. Mann−Whitney *U*-test was used for non parametric values.
Results: Although the percentages of IL-5 secreting Th2 and IL-$17A^+$IFN-γ^- Th17 cells were found similar in both groups, IL-$17A^-$ IL-22^- IFN-γ^+ Th1 and IL-$17A^-$ IL-22^+IFN-γ^- Th22 cells are significantly increased in BD patients compared to healthy subjects ($P = 0.002$ and $P = 0.028$, respectively). There was also no significant difference for the expression of CD4, CD8, CD3, CD19, however percentages of Treg ($CD4^+$ $CD25^+FOXP3^+$) cells were dramatically reduced in BD ($P = 0.000$) patients.
Conclusions: These findings revealed that increased levels of IFN-γ secreting Th1 cells as well as Th22 cells might be associated with inflammatory reactions. It has been shown that inflammatory IL-22 secreting cells are distinct from Th17 cells and Th1 cytokines inhibit IL-17 but not IL-22. In the same manner, increased levels of IFN-γ might suppress the IL-17 secretion.

P0905

Inflammatory and tissue derived dendritic cells functionally cooperate to prime Th1 skewed responses to CpG/antigen

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Purpose/Objective: Dendritic cells (DCs) harbour the unique capacity to prime and steer T cell responses. DCs are however a heterogeneous population, with multiple DC subsets displaying specialized functions. Under steady state conditions, lymph node resident and tissue derived migratory DCs can be discriminated. In case of inflammation, an additional population of inflammatory DCs (iDCs) arises. Gaining insights in the functional divergence and collaboration among DC subsets in priming Th1 responses to antigens combined with the TLR9 ligand CpG constitutes the main objective of this study.
Materials and methods: Lymph nodes of mice subcutaneously injected with OVA-488/CpG were analysed by FACS for OVA containing DC subsets and IL-12 production. Sorted DC subsets were co-cultured with CFSE labeled OT-I/II cells to assess antigen presentation. Effector T cell responses were analysed using IFN-γ elispot.
Results: Subcutaneous injection of CpG/OVA-488 results in the fast recruitment of monocyte derived $Ly6C^{hi}$ $CD11b^{hi}$ cells, resembling the phenotype of iDCs, to the draining lymph nodes. These cells gradually upregulate CD11c and MHCII, indicating they differentiate into 'bonafide' DCs, but lack CCR7, suggesting they directly migrate from the blood to the lymph node. Being the major OVA containing DC population, sorted iDCs however fail to present OVA to OT-I/II cells *ex vivo*. Tissue derived migratory DCs instead efficiently stimulate OT-I/II proliferation, showing they are the main APCs following CpG based immunization. iDCs nevertheless are crucially involved in the Th1 skewing properties of CpG, as a dramatic reduction in the effector response was observed in CCR2 KO mice, which lack iDCs. By intracellular cytokine staining, we were able to demonstrate that iDCs were by far the predominant source of IL-12 following CpG-based immunization.
Conclusions: iDCs and migratory DCs functionally cooperate to prime IFN-γ secreting CD4 and CD8 T cells following immunization with CpG/antigen. While migratory DCs are crucial for antigen presentation, iDCs in contrast provide the Th1 skewing cytokine IL-12. This dichotomy in antigen presentation and cytokine secretion thereby appears to mimic the situation observed in priming Th2 responses, where DCs present the antigen but basophils/eosinophils represent the major source of IL-4.

P906
Influence of *Salmonella* on MHC class II antigen presentation

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Purpose/Objective: Control of *Salmonella* infection requires MHC-II restricted $CD4^+$ T cell responses; however, development of adaptive immunity to *Salmonella* infection is delayed and inefficient. We have previously shown that cells harbouring *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) exhibit elevated MHC-II polyubiquitination and reduced MHC-II surface expression. The purpose of this study was to define the intracellular localisation of MHC-II down-regulated by *Salmonella* and the influence this has on antigen presentation to conventional Type A and unconventional Type B $CD4^+$ T cells.
Materials and methods: For EM, MelJuSo cells were infected with GFP-*S.* Typhimurium by SPI1-mediated invasion. Surface HLA-DR was labelled with L243 antibody at 12 h post-infection and then L243 endocytosis was assessed from 0 to 7 h. Cells were fixed, cryosectioned and labelled with 10 nm proteinA gold particles. For antigen presentation, BMDC were infected with opsonised *S.* Typhimurium. At 20 h post-infection, HEL protein or peptide and T hybridoma cells were added. T cell activation was quantified by measuring IL-2 concentration in supernatant by ELISA after 24 h. MHC-II surface expression was assessed by flow cytometry as mean L243 fluorescence of infected cells (GFP positive)/mean L243 fluorescence of uninfected cells (GFP negative) ×100.
Results: *S.* Typhimurium reduces surface expression of MHC-II by a mechanism that is independent of invariant chain directed trafficking and causes accumulation of MHC-II in multi-vesicular bodies. Reduced MHC-II surface expression in *S.* Typhimurium-infected BMDCs correlates with reduced presentation of antigen to conventional Type A T cells. In contrast, exposure of BMDCs to live *S.* Typhimurium enhances presentation of exogenous peptide to unconventional Type B T cells.
Conclusions: This data suggests that *Salmonella* infection specifically stabilises or enhances formation of the pMHC-II conformer recognised by Type B T cells, whilst the pMHC-II conformer recognised by Type A T cells is down-regulated. Type B T cells do not recognise peptide processed from protein in late endosomal compartments and therefore escape negative selection and are implicated in autoimmunity. These observations have implications with respect to the interplay between pathogens, the host immune response and autoimmunity.

P0907
Interference of the CD30-CD30L pathway reduces atherosclerosis development

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Purpose/Objective: Costimulatory molecules tightly control immune responses by providing positive signals that promote T cell activation or by transducing inhibitory signals that limit T cell responses. CD30 and CD30L are members of the TNF(R) superfamily and are involved in activation and proliferation of T and B cells, which have been implicated in the initiation and progression of atherosclerosis. In the present study, we thus aimed to determine the role of the CD30-CD30L pathway in the development of atherosclerosis.
Materials and methods: Western-type diet fed LDL receptor deficient ($LDLr^{-/-}$) mice were treated with an anti-CD30L antibody for 8 weeks.
Results: This resulted in a reduction of atherosclerotic lesion formation in the aortic root by 35%. Reduced numbers of adventitial $CD3^+$ T cells were found in anti-CD30L-treated mice, whereas no differences were observed in collagen and macrophage content of the atherosclerotic lesions. B cell and mast cell responses were also not affected upon anti-CD30L treatment. Interestingly, splenocyte proliferation was reduced with 53%, while T cell numbers were concomitantly reduced in anti-CD30L-treated mice compared with control mice. These data thus indicate that the CD30-CD30L pathway solely exerts its function via inhibition of T cell responses.
Conclusions: In the present study, we are the first to show that interruption of the CD30-CD30L pathway reduced initial atherosclerosis development by modulating T cell function.

P0908
Intravital imaging-based analysis of thromboxane A2 receptor function during physiological T cell activation

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Purpose/Objective: Thromboxane A2 (TXA2) is a prostanoid formed by thromboxane synthase using the cyclooxygenase product prostaglandin H2 as substrate. While activated dendritic cells (DCs) are able to produce TXA2, the TXA2 receptor TP is expressed by naïve T lymphocytes. *In vitro* assays have previously shown that TXA2-induced TP activation induces chemokinetic motility of T cells and decreases T cell − DC interactions, yet its *in vivo* function for controlling dynamic T cell − DC interactions in lymphoid tissue has remained elusive.
Materials and methods: The popliteal lymph node (LN) was surgically exposed in mice injected 18 h before with activated DCs, control and TP-deficient TCR tg CD4+ T cells were adoptively transferred before intravital fluorescence microscopy (IVFM) scanning. Sequences of image stacks were transformed into volume-rendered four-dimensional movies using Volocity software, which was also used for semiautomated tracking of cell motility in three dimensions and DC-T cells interactions analysis. The average track velocity and turning angles (defined as the angle between the two velocity vectors before and after a measurement time point) were calculated from the *x*, *y*, and *z* coordinates of cell centroids.
Results: Using control and TP-deficient TCR tg $CD4^+$ T cells in combination with twophoton microscopy (2 PM) of murine lymphoid tissue, we demonstrate here that TP deficiency does alter lymphocyte motility under non-inflamed conditions. In presence of activated DCs, TP-deficient T cells migrated moderately slower and with slightly larger turning angles than control T cells, although their motility coefficients remained comparable. In contrast, when activated DCs were presenting cognate peptide, the migration velocity of peptide-specific $CD4^+$ T cells was markedly reduced in the absence of TP as compared to wild type cells, irrespective of the antigenic dose. This reduction in T cell motility correlated with prolonged interactions times between TP-deficient $CD4^+$ T cells and DCs.
Conclusions: In sum, our data identify TXA2-induced TP signaling as a negative regulator of T cell − DC interactions, which may contribute to prevent excessive immune reactions and autoimmunity.

P0909

Investigating human dendritic cell localization and migrating behaviour in three-dimensional models of lung mucosa

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Purpose/Objective: Tissue homeostasis is a dynamic process dependent on the cooperation between immune cells, such as dendritic cells (DC), and tissue specific cells, including epithelial cells and stromal cells (i.e. fibroblasts). Within lung, DC mainly associates with the epithelial layer and there is evidence of DC regulation by the epithelium and that epithelial dysfunction leads to overzealous immune cell activation. However, dissecting basic mechanisms of DC interactions with epithelial cells in human tissue is difficult. Here, we present protocols to prepare three-dimensional collagen matrices with fibroblasts, establish epithelial layers and image and quantify DC activities within lung mucosa using time-lapse fluorescence microscopy.
Materials and methods: Retroviral vectors were used to generate stable GFP epithelial cells and orange fibroblasts. DC were labelled with a far red cell tracker dye before implantation into the tissue model. The model is created on Transwell inserts to allow culture at the air-liquid interface. Monocyte-derived DC were implanted in between the fibroblasts and epithelial layer. Before imaging, models were stimulated with 100 ng/ml LPS. Live imaging analyses were performed to study DC distribution and migratory behaviour in the tissue model using both confocal and multiphoton microscopy.
Results: The tissue model recapitulates key anatomical and functional features of lung mucosal tissue, including a stratified epithelial cell layer, deposition of extracellular matrix proteins and the production of tight- and adherence- junction proteins. Labelling of fixed tissue model sections and imaging of live tissue models revealed that DC distribute in close association with the epithelial layer. Time-lapse fluorescence microscopy revealed altered distribution of DC with a more a frequent appearance of DC at the apical side of the epithelium when the model was stimulated with LPS.
Conclusions: This novel tissue model provides a tool well suited for a wide range of studies, such as elucidating incompletely defined pathways controlling homeostatic activities of human DC in mucosal tissue, as well as DC responses to pathogen-induced inflammation.

P0910

Investigating the role of cutaneous dendritic cells in T-cell mediated immunopathology

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Purpose/Objective: Severe and chronic immunopathology mediated by T cells is common to many cutaneous diseases. An important example is graft-versus-host-disease (GVHD). In MHC-mismatched models of bone marrow transplantation, host antigen presenting cells that survive the conditioning regime have been shown to be essential for priming GVHD. We have previously shown that inflammation within peripheral tissues is critical to the recruitment and function of effector T cells (Chakraverty *et al.* JEM 2006). To assess whether Langerin+ dendritic cells (DC) have a role regulating this inflammatory checkpoint, we developed a model of cutaneous GVHD where host Langerin+ DC can be selectively depleted. Minor histocompatibility antigen mismatched models were also developed to test the role of Langerin+ DC in more clinically relevant models of GVHD.
Materials and methods: MHC-mismatched mixed chimeras (MC) were generated by reconstituting irradiated Langerin-DTR (or B6) recipients with T cell depleted bone marrow (BM) from B6 (or Langerin-DTR) and Balb/c mice. Eight weeks later, MC were injected with Balb/c T cells. Localized skin GVHD was induced by topical application of Imiquimod, a TLR7 agonist. Langerin+ DC were depleted by i.p. injection of diphtheria toxin. In another model, male Langerin-DTR mice were lethally irradiated and reconstituted with female B6 BM and either CD4 (Marilyn) or CD8 (MataHari) cells transgenic for HY-specific TCR. Depletion of Langerin+ DC was induced by i.p. DT.
Results: Depletion of Langerhans cells (LC) in MC resulted in significant reductions in cutaneous GVHD. However, the absence of host LC had no effect on the priming or frequency of donor CD8 T cells found in skin or draining lymph nodes. Rather, the capacity of epidermal CD8 T cells to generate IFN-γ, TNF-α and TRAIL was significantly reduced. When host Langerin+ dermal DC but not LC were depleted, there was no effect on the accumulation of donor CD8 cells within skin draining lymph nodes or within the epidermis and no effect on the severity of cutaneous GVHD. In the HY-mismatched model of BMT, depletion of Langerin+ DC reduced recruitment of HY-specific CD4 or CD8 cells to skin.
Conclusions: Langerin+ DC contribute to CD8 T cell mediated immunopathology in the skin. In a major mismatch model of GVHD, host LC were required to 'license' T cells for full effector function. In a minor mismatch model of GVHD, host Langerin+ DC are required for the accumulation of antigen specific CD4 and CD8 cells in the skin.

P0911

Irap is required for normal phagosome maturation in dendritic cells

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Purpose/Objective: Among phagocytes, dendritic cells (DCs) are characterized by mild acidification and delayed maturation of their phagosomes, features that have been demonstrated to be essential for an efficient MHC class I cross-presentation. Known molecular mechanisms contributing to delayed maturation of DC phagosomes include Sec22-mediated NOX2 mediated alkalinization of phagosomes and Sec22-mediated ERGIC recruitment to them. We have recently identified a previously unappreciated cellular compartment that is massively recruited to DC phagosomes and required for antigen cross-presentation. This compartment corresponds to regulated storage endosomes described in other cell types and contains Insulin Responsive AminoPeptidase (IRAP), which acts as a trimming peptidase for cross-presented peptides. Here we analyzed the contribution of IRAP+ storage endosomes to phagosome maturation in DCs.
Materials and methods: Phagosomes obtained from wt and IRAP deficient DCs were analyzed by confocal microscopy, immunoblot and phagoFACS with respect to the kinetics of their maturation, phagosomal cargo degradation and intra-phagosomal pathogens killing.
Results: IRAP-deficient DCs showed accelerated phagosome maturation as documented by premature loss of early endosomal markers such as EEA1 and Rab14, and accelerated acquisition of phagolysosome markers such as Lamp1 and V-ATPase. This was associated with accelerated degradation of phagocytosed antigen, enhanced acidification of endosomes and increased killing of internalized fungal cells and bacteria.
Conclusions: Our results suggest that fusion with the specific endosomal compartment containing IRAP is required for normal phagosome maturation in DCs.

P0912

Kinetics of codominant MHC protein expression and implications for disease associations

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Purpose/Objective: Genotype/phenotype relationships, such as MHC associations with autoimmune disease, remain a puzzle for biomedicine in the postgenomic era. Extensive polymorphism of codominantly expressed MHC alleles diversifies peptide presentation to T cells, but some alleles increase autoimmune disease risk. Some of these associations have been blamed on protein misfolding (HLA-B27 in ankylosing spondylitis) or increased turnover (H2-A^{g7} in NOD mice). However, little is known about how MHC polymorphism affects protein turnover. This was investigated here.

Materials and methods: A stable isotope labelling approach was developed to this end. Human APCs or mice were labelled with heavy water (^2H_2O), cells were harvested after various times, and folded MHC proteins immunoprecipitated. After SDS-PAGE and tryptic digestion, ^2H incorporation was tracked by LC/MS and used to calculate fractional protein synthesis. To control for cell proliferation, fractional DNA synthesis was quantified simultaneously by GC/MS analysis of ^2H incorporation into purine deoxyribose.

Results: The turnover rates of folded MHC proteins differed by class and isotype and were strongly affected by cellular background. In human myeloid APC lines and MoDCs, HLA-C molecules turned over faster than HLA-B; HLA-A turnover rates varied between individuals. HLA-DR, but not MHCI turnover was shut down by LPS activation of MoDCs. HLA-B and DR turnover were very slow in EBV-transformed B cells. Folded HLA-B27 molecules had similar turnover rates as other B alleles. *In vivo,* murine splenic MHCII protein turnover was faster in DCs than in B cells. Allelic differences in MHC protein turnover were slight. A^{g7} turnover differed from A^d or E^d by <20% overall, although A^{g7} exhibited kinetic heterogeneity in B cells.

Conclusions: Cell type and activation state, as well as MHC isotype, regulate the turnover rates of folded MHC proteins in physiological APCs. In contrast, the impact of allelic polymorphism in physiological APCs is minor, limiting the possible roles of MHC protein turnover in autoimmune pathogenesis. Stringent conservation of protein life span between alleles suggests critical roles in codominant expression and function of MHC proteins. The approach developed here will be generally useful for probing the impact of structural polymorphism on protein turnover, and its relevance to disease, *in vivo*.

P0913

Lactoferrin modulates immune responses induced by chemical allergens

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Purpose/Objective: Lactoferrin (LF) is an 80 kDa glycoprotein from the transferrin family. It is present in mammalian epithelial secretions and secondary neutrophil granules. LF is well known as an anti bacterial protein due to its ability to sequester iron as well as its potential to bind lipopolysaccharide. Recently LF has emerged as an immunomodulatory molecule with reports suggesting that it can exert both immunostimulatory and immunosuppressive functions. For example, in both man and mouse we have shown that the homologous LF inhibits Langerhans cell (LC) migration secondary to effects on TNF-α. The purpose of these investigations is to further characterise the action of LF *in vivo*.

Materials and methods: BALB/c strain mice were treated topically on the dorsum of both ears with 0.5 μg of human native milk LF in aqueous cream or cream alone. Two hours later the same site was treated with chemical contact allergen: either 0.5% oxazolone (Ox) or 1% 2,4-dinitrochlorobenzene (DNCB). Four to 24 h thereafter ears were excised, split into dorsal and ventral halves, epidermal sheets were prepared, and stained for LC enumeration using fluorescein isothiocyanate labelled anti-major histocompatibility complex class II antibody.

Results: The resting levels of LC in naïve BALB/c female mice were approximately 1100 LC/mm^2. Consistent with previous experience, topical exposure (4 h) to either allergen resulted in a significant decrease in LC frequency (25−30%) indicating the proportion of LC that appear to be available for rapid mobilization. Prior treatment with LF (2 h) significantly inhibited LC migration triggered by exposure to Ox (4 h) (3% and 24% migration in presence and absence of LF, respectively) but was without effect on DNCB-induced LC mobilization (17% and 21% migration in presence and absence of LF, respectively). The effects of LF on Ox-induced LC migration were reproducible but relatively transient, such that significant Ox-induced reductions in LC frequency (12% and 17%) were recorded after 6 and 24 h in the groups of animals pre-treated with LF.

Conclusions: Taken together, these results demonstrate that heterologous (human) LF functions as an immunomodulatory protein following topical exposure in mice. Furthermore, the effects of LF are allergen-specific and indicate that Ox- and DNCB-induced LC migration may have differential requirements for TNF-α.

P0914

Leishmania major lysate as source for potential immunogenic antigens

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Purpose/Objective: Cutaneous leishmaniasis (CL) is caused e.g. by the parasite *Leishmania major*. It is known that healing of CL requires interferon (IFN)γ release by both antigen-specific $CD4^+$ Th1 and $CD8^+$ Tc1 cells. Nonetheless, an effective vaccine does not exist at present, even though its development is of highest interest due to increased morbidity of affected individuals with co-infections or immunosuppression. Therefore, we aim to identify and characterize *L. major*-specific immunogenic proteins from soluble *Leishmania* antigen (SLA) with proven efficacy during vaccination approaches.

Materials and methods: First, differential centrifugation was performed to partition SLA into its components (nuclei, mitochondria, membrane proteins, soluble proteins). Next, to further break-down the complexity of SLA, we focused on the soluble proteins, which we fractionated by using combinations of different anion exchange chromatography columns. Eluted fractions were tested for their T cell restimulating ability (cytokine release, proliferation) *in vitro* using draining lymph node (dLN) T cells isolated from resistant, infected C57BL/6 mice.

Results: dLN cells restimulated with SLA typically show a dominant Th1/Tc1 cytokine profile. Interestingly, all components obtained after differential centrifugation induced reactivity similar to total SLA. After combined chromatographic fractionation of the soluble proteins, we identified several fractions that induced high IFNγ levels compared to unstimulated controls. As expected for exogenous antigens, $CD4^+$ T cell reactivity played an important role, as the fraction-specific

proliferation of $CD4^+$ dominated over the expansion of $CD8^+$ T cells. IFNγ-releasing fractions will next be analyzed by label-free mass spectrometry for protein content.
Conclusions: Since immunization studies of mice showed promising results with selected recombinant *Leishmania* proteins combined with adjuvant, identification of additional immunogenic antigens *in vitro* and *in vivo* may contribute to a better understanding of T cell-mediated protection against CL; however, better antigens need to be characterized that mediate full protection.

P0916

Mutant Gimap5 derived from diabetes-prone BB rats causes selective enrichment of Th1/Th17 cells and an altered TCR repertoire in Lewis rats

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Purpose/Objective: Deficiency of the *GTPase of the immunity-associated protein 5* (Gimap5) causes severe T cell lymphopenia and is responsible for the development of autoimmune Type 1 Diabetes in BB rats with the MHC haplotype $RT1^U$. Gimap5 in these rats carries a single point mutation, resulting in the expression of a truncated, non-functional protein. Consequently, peripheral CD8+ and CD4+ T lymphocyte numbers are dramatically diminished due to increased death of recent thymic emigrants. This appears to explain the selective enrichment of autoreactive T cells as indicated by an oligoclonal expansion of the remaining T cells. Interestingly, Gimap5 deficiency in PVG rats also leads to T-lymphopenia and autoimmunity, although Gimap5 deficient rats on this genetic background develop eosinophilic bowel disease rather than Type 1 diabetes. Moreover, Gimap5 polymorphisms in humans are linked to systemic lupus erythematosus and these patients are also lymphopenic.
Materials and methods: To further characterize Gimap5 function, we have backcrossed the mutant Gimap5 allele to Lewis rats harbouring the MHC haplotype $RT1^{B/D}$. By using speed congenics we were able to obtain a 99% pure Lewis background within 5 generations.
Results: Gimap5 deficient Lewis rats develop T lymphopenia in the periphery and show an altered TCR repertoire. Whereas the V beta chain usage in the thymus is unchanged, some V beta chains are overrepresented on peripheral T cells of Gimap5 deficient Lewis rats as compared to controls. However, the affected V beta chains are not identical to the ones altered in BB rats. The few remaining T cells in the Gimap5 deficient Lewis rats exhibit an activated phenotype as indicated by high levels of CD134 and CD11a expression and low levels of CD62L. Furthermore, the relative number of IFNγ and IL-17 producing T cells is increased while the frequency of regulatory T cells is not affected. Although up to now we did not observe any spontaneous disease development, Gimap5 deficient rats suffer from a fulminant disease course after active EAE induction.
Conclusions: Collectively, our data indicate that Gimap5 is important for T cell survival and adjustment of the different T cell subsets and that its absence results in an altered TCR repertoire and a disproportionally high frequency of Th1/Th17 cells.

P0917

New approach in EAE treatment, part I: the application of dendritic cells stimulated with Trichinella spiralis antigens results in dose dependent effect

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Purpose/Objective: Helminth infection has a potent systemic immunomodulatory effect on the host immune response, which also affects the development of autoimmune diseases. Our model of combined *Trichinella spiralis* (*T. spiralis*) infection and experimental autoimmune encephalomyelitis (EAE) in Dark Agouti (DA) rats demonstrated a significant reduction in EAE severity in infected animals. The results indicated that the final muscle stage of parasite infection can affect the outcome of a particular autoimmune disease in a manner beneficial to the host. Recently, we have achieved to create immune status characteristic for the live infection by *in vivo* application of dendritic cells (DCs) stimulated with excretory secretory (ES L1) products of *T. spiralis* muscle larvae. The aim of current research was to investigate the effect of DCs educated with ES L1 on the development and course of EAE.
Materials and methods: Different doses of DCs (0.5×10^6, 1×10^6, 5×10^6) stimulated with ES L1 were administered i.p. into naïve DA rats 7 days before EAE induction. The clinical signs of the disease were monitored for 25 days. We examined the production of IFN-γ, IL-17, IL-4, IL-10 and TGF-β, as well as the presence of $CD4^+$ CD25+Foxp3+ cells at the end of the observation period, both at the level of spinal cord and at systemic level.
Results: The applied doses differently influenced the disease development. The recipients of 0.5×10^6 DCs had similar clinical sings as control animals (received DCs cultivated in medium only before EAE induction). Doses of 1×10^6 and 5×10^6 DCs reduced the severity of EAE as judged by lower maximal clinical score (1.8 ± 1.3 and 1.6 ± 1.5 both versus control 2.4 ± 0.6), cumulative index (0.26 ± 0.2 and 0.22 ± 0.2 both versus control 0.55 ± 0.2), duration of illness (4.2 ± 2.4 and 3 ± 3.1 both versus control 9.4 ± 3.7) and degree of mononuclear cell infiltration in central nervous system (CNS). The production of IL-17 was significantly decreased, while the production of IL-10 and TGF-β was greatly increased in animals treated with ES L1 stimulated DCs compared to controls. The proportion of regulatory T cells was significantly higher in recovered recipients of ES L1 stimulated DCs.
Conclusions: Novel approach in treatment of EAE by educated DCs gave promising results and brought us closer to the establishment of a new therapy.
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P0918

New approach in EAE treatment, part II: Mechanisms underlying the beneficial effects of dendritic cells stimulated with Trichinella spiralis antigens

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Purpose/Objective: Parasitic nematode *Trichinella spiralis (T. spiralis)* exert immunomodulatory effect on the host immune response through excretory-secretory products (ES L1) released from the encysted muscle larvae. Rat bone-marrow derived dendritic cells (DCs) stimulated with ES L1 antigens acquire semi-matured status and induce Th2 and regulatory responses when applied into naïve Dark Agouti (DA)

rats. Moreover, these cells were able to ameliorate experimental autoimmune encephalomyelitis (EAE) when applied 7 days before EAE induction. This study is focused on the mechanisms underlying observed beneficial effects of DCs stimulated with ES L1 antigens on the development and course of EAE.

Materials and methods: DCs stimulated with ES L1 antigens (5×10^6) were applied to DA rats 7 days before the induction of EAE. We analyzed the kinetics of production of IL-4, IL-10, TGF-β, IFN-γ and IL-17 by cells harvested from spleens and CNS throughout the disease, ie in inductive (day 8 p.i.), effector (day 15 p.i.) and recovery phase (day 25 p.i.). The proportion of Foxp3+ T cells at each time point was estimated.

Results: Our results indicated that educated DCs altered the immune response responsible for the development of EAE via increased production of IL-4, IL-10 and TGF-β, and decreased production of IFN-γ and IL-17, both on systemic level and the level of target organ. Significant increase in the proportion of $CD4^+$ CD25+Foxp3+ cells was found among spleen cells and CNS infiltrates from DA rats treated with ES L1 stimulated DCs before EAE induction compared to immunized, non-treated controls.

Conclusions: Our results show that ES L1 antigen stimulated DCs are able not only to provoke, but also to sustain anti-inflammatory and regulatory responses regardless of EAE induction, which results in amelioration of EAE, or even protection from the disease.

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P0919

Novel Ap4A second messenger signaling in Bone Marrow-derived Dendritic Cell (BMDC) differentiation, maturation and function

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Purpose/Objective: Diadenosine tetraphosphate, Ap4A acts as a second messenger in the Lysyl-tRNA synthetase (LysRS) signaling pathway. It is produced by Phosphorylated LysRS and in turn it activates a family of microphthalmia-associated transcription factors (MITF). Tartarate-resistant acid phosphatase 5 (TRACP5) expressed in osteoclasts and dendritic cells (DC) is transcriptionally regulated by MITF. We aim to characterize the Ap4A second messenger signaling pathway in BMDC.

Materials and methods: BMDCs were isolated and cultured with Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) and activated with lipopolysaccharide (LPS), GM-CSF and Receptor Activator for Nuclear Factor kappa B Ligand (RANKL). Whole cell lysate was collected for analysis by SDS-PAGE. Total RNA was isolated, converted to cDNA and analyzed by SYBR-green real-time PCR. Ap4A levels were analyzed by hydrolysis of Ap4A in an ATP assay. The Dendritic cell line, JAWS II, was used as an *in vitro* model to study the effects of LysRS overexpression in DC. Mutation of Serine 207 into aspartate mimics the phosphorylated state of LysRS on residue 207, thus JAWS II was transfected with 3 different constructs, LysRS WT, S207A that act as a control and S207D which represents overexpression.

Results: We observed that mRNA expression of IL-23p19 in BMDC stimulated with LPS, GM-CSF and RANKL resulted in a 1000-fold increase. DC activation also induced a 27-fold rise in intracellular Ap4A levels and a 3-fold rise in MITF mRNA. TRACP5 gene and protein expression were also up regulated in LPS/GM-CSF/RANKL activated BMDCs. We found that upon activation with LPS, GM-CSF and RANKL, JAWS ll cell line transfected with LysRS S207D show similar IL23p19 mRNA and TRACP5 expression profile to BMDC when activated.

Conclusions: Our results show that activation of BMDC by LPS/GM-CSF/RANKL results in MITF-specific gene activation, suggesting that the Ap4A signaling pathway functions in DC as a mechanism for gene regulation, and may serve as a novel DC signaling pathway. Further investigations in functional significance of the pathway are currently underway.

P0920

Phenoypical characterization and isolation of a subpopulation of myeloid cells from swine peripheral blood: new method to isolate plasmacytoid dendritic cells

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Purpose/Objective: Our study aims to isolate a subpopulation phenotypically identified as $CD11R3^-$ $CD172a^{lo}$ and investigate a possible relationship with pDC subset.

Materials and methods: PBMC from healthy pigs were collected and a magnetic depletion using negative selection for $CD3^-$ $CD163^-$ cells was performed, discarding a major part of the lymphocytic and monocytes in the population. Secondly, the subpopulation obtained was characterized for different surface markers and further isolated through sorting with FACSAria Sorter (BD Becton, Dickinson). The subpopulation obtained was evaluated by electron microscopy (EM) Jeol 1400. Also, a functional analysis was performed to test the ability of these cells to be stimulated either with TLR agonist (Poly:IC, R837 or CpG) or infected with H3N2 at MOI 0.01. Activation was quantified for IFNa production by ELISA.

Results: Cells under study revealed a defined rounded to oval shape with a centered nucleus by EM. These cells were $CD3^-$, $CD163^-$, $CD172a^{lo}$, $CD11R3^-$ and $CD4^+$. Functional assays also suggest that this population could be pDC.

Conclusions: The subpopulation $CD172a^{lo}$ $CD11R3^-$ isolated through sorting showed morphological and phenotypical characteristics of the pDC subpopulation.

P0922

Preferential HLA-DRB1*11 dependent presentation of ADAMTS13 derived peptides in autoimmune thrombotic thrombocytopenic purpura

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Purpose/Objective: Autoantibodies directed against ADAMTS13, a plasma metalloproteinase, prohibit the processing of VWF multimers initiating a rare and life-threatening disorder called acquired thrombotic thrombocytopenic purpura (TTP). At present the cause of antibody formation in these patients is unknown. Recently HLA-DRB1*11 has been identified as a risk factor for the development of acquired TTP. This finding indicates that ADAMTS13 derived peptides are presented to CD4+ T cells by antigen presenting cells. We have recently identified that ADAMTS13 is endocytosed by immature monocyte derived dendritic cells in a mannose receptor dependent manner. The aim of the current study is to directly identify naturally ADAMTS13-derived peptides presented on MHC class II alleles.

Materials and methods: Dendritic cells from a panel of both HLA-DRB1*11 positive and negative donors were pulsed with antigen.

Subsequently the HLA-DR-peptide repertoire was analyzed by means of affinity-purification and mass spectrometric peptide sequencing.
Results: Interestingly, at low antigen concentration only DR11-positive donors presented CUB1-2 derived ADAMTS13 peptides. Increasing the concentration of the antigen lead to the identification of a higher number of ADAMTS13 derived peptides by both HLA-DRB1*11 positive and negative donors. Although the presented peptides belonged to several ADAMTS13 domains, inspection of the peptide-profiles revealed that antigenic 'core' peptides, originated from the CUB1-2 domains of ADAMTS13, were presented with a higher efficiency compared to other peptides.
Conclusions: These findings show that antigen presenting cells, in particular HLA-DRB1*11 positive cells, preferentially present antigenic 'core' peptides derived from the CUB1-2 domains of ADAMTS13. We hypothesize that functional presentation of these peptides on HLA-DRB1*11 contributes to the onset of acquired TTP by stimulating low affinity self-reactive CD4+ T cells that have escaped negative selection in the thymus.

P0923

Protective HLA Molecules Determine Infection Outcome by Preferential Presentation of Peptides From Conserved Hepatitis C Proteins

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Purpose/Objective: Hepatitis C virus infections (HCV) affect worldwide more than 170 million people. While the majority of these individuals are chronically infected, some clear the infection rapidly. Host factors seem to play a key role in the clearance of HCV, among them the human leukocyte antigen (HLA) class I molecules. Particular HLA molecules, e.g. B*27 and B*57, are associated with viral clearance.
Materials and methods: To get a better understanding of the mechanisms behind these associations, we analyzed the HLA-restriction of both experimentally verified and *in silico* predicted HCV epitopes with respect to the sequence variability of HCV proteins, which was estimated using publicly available HCV sequences.
Results: Core, NS5B and NS3 are the most conserved proteins of HCV, while E1 is the most variable. HLA molecules associated with HCV clearance preferentially present cytotoxic T cell (CTL) epitopes from conserved HCV proteins: especially NS5B is significantly enriched in epitopes presented by HLA-B*57 and HLA-B*27. In contrast, none of the known susceptible HLA molecules, i.e. HLA molecules associated with HCV persistence, have a similar preference.
Conclusions: Our analysis suggests that by targeting the most constrained – and thereby conserved – proteins of HCV, ÒprotectiveÓ HLA molecules reduce the potential of HCV to escape the cytotoxic T cell response of the infected host.

P0925

Quantifying the contribution of polymorphic residues to peptide binding of HLA molecules

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Purpose/Objective: HLA genes are known to be the most polymorphic genes in the human genome. Almost all polymorphic residues are located in the peptide binding groove, resulting in different binding preferences. Using the structural data it is possible to infer the proximity of the polymorphic site to the peptide, and thus determine which sites might be in contact with the peptide However, to date it has not been possible to quantify the contribution of each polymorphic residue to the actual peptide binding.
Materials and methods: We make use of an in silico peptide-MHC binding predictor, netMHCpan-2.4. This method combines the information on the polymorphic sites in the binding groove (the so called HLA pseudo-sequence) with the measured peptide-HLA binding affinities, and was shown to be a highly accurate predictor of peptide-HLA binding affinity. We first predict the binding affinity of 100.000 natural peptides for the common HLA molecules. Next, we simulate point mutations in the HLA molecules by generating new sequences that differs only one position with a 'real' HLA molecule, and predict the binding affinity of the natural peptides the same way. We then focus on the top 1% best binding peptides as most likely binders, and calculate the repertoire overlap between the 'real' HLA molecule and the mutated one.
Results: The repertoire overlap, as defined here, allows us to quantify the functional effect of polymorphic residues. Our results show that mutations in a few positions, e.g. 116, effect the HLA peptide repertoire much more than the in other positions, and that the contribution of the polymorphic residues to the peptide binding is very different. Moreover, our analysis show clear differences between HLA-A and HLA-B molecules, which might shed light into the functional differences between these two HLA loci.
Conclusions: Both the position and the amino acid substitution are important for changing the binding motif. The HLA-B backbone is more sensitive to substitutions, i.e. a substitution could make a new binding preference. Next to recombination this could be a reason why we see more polymorphism in the B locus compared to the A locus.

P0926

Rapamycin prevents the onset of EAE by modulating proliferation and migration of pathogenic effector T-cells

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Purpose/Objective: Rapamycin is an immunosuppressant drug used to prevent allogenic transplant rejection. Due to its powerful immunosuppressive effects, it has been suggested that rapamycin may be effective in treating autoimmune diseases such as MS. Utilising the animal model of MS, experimental autoimmune encephalomyelitis (EAE), studies have shown that rapamycin is able to ameliorate the clinical signs of EAE, in both rats and mice. Standard EAE models provide limited information as to the mechanism of potential therapeutic drugs such as rapamycin. To overcome this we have developed an 'MBP tracker' CD4+ve T cell transfer EAE model which allows us to specifically track pathogenic T cells and therefore help us to determine the mode of action of a particular drug, *in vivo*. Using the MBP Tracker model, we have shown that rapamycin appears to prevent the clinical signs of EAE by preventing proliferation and migration of pathogenic effector cells.
Materials and methods: Traceable, TCR transgenic CD4+ve cells specifically responsive to a myelin basic protein (MBP) peptide epitope were transferred to non-transgenic host mice by i.v injection prior to immunisation with MBP peptide in adjuvant. Tissues were harvested and processed for subsequent analysis of cells by flow cytometry or ELISA.
Results: Rapamycin was able to prevent the onset of disease in MBP tracker model. Analysis by flow cytometry shows that there are fewer CD4+, pathogenic donor cells present in both the spleen and CNS of mice treated with rapamycin, compared to vehicle. There are also changes in the expression of cell surface markers such as CD62L.
Conclusions: Our data suggest that the main mode of action by which rapamycin is able to suppress the clinical signs of EAE is by modulating the clonal expansion of pathogenic T cells and their ability to migrate

into the CNS. This newly developed MBP-tracker EAE model not only provides efficacy data, but by allowing us to specifically track the cells capable of causing disease, we are able to obtain mode of action data on potential therapeutic targets.

P0927

Reactive carbonyl adduction of proteins primes TH2 responses by enhanced MHCII/peptide complexes on dendritic cell surface

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Purpose/Objective: Oxidation has been linked to altered protein immunogenicity, possibly by chemical adductions that can serve as ligands for pattern receptors of innate immunity. We have previously demonstrated that reactive carbonyls (RCs), commonly generated on proteins during oxidation, serve as a common group of damage associated molecular patterns (DAMPs) to enhance TH2 type immune responses. However, no molecular mechanism(s) has yet been put forward. This work explores pathways through which RCs may confer their immunomodulation.
Materials and methods: Proteins were carbonylated using biologically relevant aldehydes, and the modifications were characterized using carbonyl essays, mass spectrometry, chromatography, and electrophoresis. Antigen presenting cells, as well as reporter cell lines, was pulsed with modified and unmodified antigens and antigen uptake and presentation, along with signs of stimulation, were assayed in the presence of specific blockers. Wild type and knock-out mice for key immune molecules were immunized and their B and T cell responses analysed.
Results: *In vitro* pulsing and *in vivo* cell transfer experiments established that dendritic cells (DCs) are sufficient to confer immunomodulatory effects of RC-modified antigens. While pulsed DCs showed no signs of conventional activation, they expressed higher density of MHCII-peptide on their cell surface leading to enhanced priming of T cells *in vitro* and *in vivo*. This effect was blocked by pan-blockers of scavenger receptors (e.g. dextran sulphate), but was increased by blocking or knocking down two RC-binding scavenger receptors, SRA and CD36.
Conclusions: RC adduction of antigens increases their uptake/processing/presentation, without a marked activation of DCs, leading to enhanced T cell priming with a TH2 polarization effect. While the exact PRR(s) responsible for this effect is still under investigation, our data confirms the two main candidate scavenger receptors SRA and CD36 as binding but rules out their involvement in the observed immune enhancing effects of RC adduction. Given the prevalence of carbonylated self and non-self antigens in autoimmune, inflammatory, and allergic conditions, characterization of their putative receptor(s) and mode of immune enhancement is of universal importance.

P0928

Regulation of Flt3-L availability for dendritic cell development

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Purpose/Objective: Flt3-L is a key cytokine instructing development of conventional DCs (cDCs). Although it is know that the size of the DC compartment regulates the availability of Flt3-L, very little is understood regarding tissue distribution and regulation of Flt3-L. The aim of this study was to characterise the source of Flt3-L required for cDC differentiation and to define the molecular mechanisms by which cDCs regulate Flt3-L availability.
Materials and methods: Flt3-L expression was investigated using BAC transgenic reporter mice in which eGFP and luciferase are expressed under the control of the *flt3l* promoter (FL-GL mice).
Results: Using a novel Flt3-L reporter-mouse strain, we found ubiquitous Flt3-L expression. All cells and organs investigated expressed Flt3-L, likely ensuring optimal cDC generation across different organs. Restricting Flt3-L expression to the hematopoietic or non-hematopoietic compartments did not result in a significant alteration in the frequency of DC precursors (MDPs, CDPs, pre-cDCs). However, we observed organ-specific differences in the contribution of different cellular compartments to final cDC differentiation. Flt3-L in all non-lymphoid organs was largely derived from non-hematopoietic cells and, accordingly, this source was mandatory and sufficient for the $CD103^+$ cDC compartment in lung, kidney and pancreas. Liver was exceptional in that hematopoietic and non-hematopoietic sources of Flt3-L were both required for normal cDC generation. The requisite of Flt3-L for generating $CD103^-$ $CD11b^{hi}$ cDCs was variable depending on the non-lymphoid organ investigated, possibly reflecting heterogeneity in this cDC compartment. Regarding cDC development in lymphoid organs, hematopoietic and non-hematopoietic cells equally contributed to splenic Flt3-L, although production by the former was necessary and sufficient for normal splenic cDC development indicating that the source rather than the total amount of splenic Flt3-L is dominant for splenic cDC development. In addition, data on regulation of Flt3-L expression by cDCs will be presented.
Conclusions: Flt3-L is ubiquitous across mouse organs and cells. The cellular source of Flt3-L instructing cDC development varies significantly depending on the organ in question. These results highlight different strategies adopted to optimise cDC development across organs presenting such different microenvironments.

P0929

Remodeling of lipid metabolism by IL-17A in human dendritic cells

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Purpose/Objective: Our laboratory has previously shown a crucial role of IL-17A in a rare disease of unknown etiology called Langerhans cell hystiocytosis (LCH). LCH is characterized by the accumulation of atypical dendritic cells resembling skin DC (LCH-DC), but also other immune cells including multinucleated giant cells (MGC) to form aggressive granulomas that destroy the tissues. In LCH, IL-17A is synthesized by LCH-DC and MGC. IL-17A induces survival of DC and their fusion leading to MGC *in vitro*, as observed in granuloma *in vivo*. Exogenous recombinant IL-17A shows the same effects on DC from healthy donors which do not produce autocrine IL-17A (Coury *et al.* Nat Med, 2008). The objective of this study was to further understand the molecular mechanisms underlying IL-17A-mediated biological effects on DC.
Materials and methods: We used Affymetrix microarrays to compare the gene expression profile of IL-17A-treated DC and LCH-DC to that of DC from healthy donors. Transcriptome analysis suggested a complete remodelling of lipid metabolism in response to IL-17A in DC. Lipid accumulation in IL-17A-treated DC was confirmed by Oil Red-O, a lipid-soluble dye which stains neutral lipids. The exact nature of the lipids present in IL-17A-treated DC was determined by high performance thin layer chromatography after lipid extraction.
Results: Transcriptome analysis, validated at the mRNA level by qPCR, showed that expression of enzymes, transporters and other proteins involved in lipid storage and metabolism, were profoundly

affected in IL-17-treated DC and LCH-DC compared to DC from healthy donors. In agreement with transcriptome data, we found that both DC from LCH patient and IL-17A-treated DC from healthy donors accumulated huge amounts of lipid droplets in their cytoplasm. Interestingly, levels of phospholipids, triglycerides, cholesteryl esters and cholesterol were all increased in IL-17A-treated DC.
Conclusions: We have shown for the first time a link between IL-17A and lipid metabolism in DC. This is of major interest, not only in LCH but also in other chronic inflammatory diseases.

P0932
Secretory leukocyte proteinase inhibitor (SLPI) competent DNA deposits are potent stimulators of plasmacytoid dendritic cells in psoriasis

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Purpose/Objective: Secretory leukocyte proteinase inhibitor (SLPI) is a well-established inhibitor of serine proteases such as human neutrophil elastase (HNE) and a NF-κB regulatory agent in immune cells. Here, we report that SLPI plays a previously uncharacterized role in regulating activation of plasmacytoid dendritic cells (pDCs). As the main source of interferon type I (IFNI), pDCs are crucial contributors to inflammatory and likely wound healing responses associated with psoriasis. The mechanisms responsible for activation of pDCs in psoriatic skin are therefore of substantial interest.
Materials and methods: Immunohistochemistry, flow cytometry, magnetic cell sorting, ELISA.
Results: We demonstrate that in lesional skin of psoriasis patients, SLPI together with its enzymatic target HNE and DNA, is a component of neutrophil extracellular traps (NETs). Whereas SLPI-positive neutrophils and NETs were found to colocalize with pDCs in psoriatic skin, a mixture of SLPI with neutrophil DNA and HNE induced a marked production of IFNI by pDCs. IFNI synthesis by stimulated pDCs was dependent on intracellular DNA receptor TLR9.
Conclusions: SLPI may contribute to psoriasis by enabling pDCs to sense extracellular DNA and produce IFNI.

P0934
Skin pathology in Wiskott-Aldrich syndrome mice is caused by hyperactive CD8$^+$ T cells and increased cross-presentation by DCs

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Purpose/Objective: Wiskott-Aldrich syndrome (WAS) is a primary immunodeficiency caused by loss-of-function mutations in the WAS protein (WASp). WASp coordinates upstream receptor signaling to changes in the actin cytoskeleton in hematopoietic cells. A major clinical complication for WAS patients is development of eczema. Because WAS is considered a 'cell-trafficking disease' in which all hematopoietic cells show decreased migratory, adhesive, and receptor-mediated responses, it remains elusive why WAS patients develop eczema, caused by an over-active immune system.
Materials and methods: Wildtype and WAS protein-deficient (WASp KO) mice were epicutaneously challenged with the allergen Der p 2 to induce eczema or infected intradermally in the ears with *Leishmania major* to induce a robust skin inflammation. To investigate the capacity to cross-present, wildtype and WASp-deficient dendritic cells were isolated from spleen and pulsed with OVA and stimulated with LPS, and afterwards co-cultured with OT-I CD8$^+$ T cells.
Results: Der p 2 challenge of wildtype mice induced eczema with increased epidermal hyperplasia and egress of Langerhans cells from the skin. WASp KO mice had increased accumulation of mature DCs in the skin and, in contrast to wildtype cells, Der p 2 induced expansions of WASp KO CD8$^+$ T cells that produced IFNγ.

Wildtype and WASp KO mice responded vigorously to infection with *L. major* with massive cellular infiltration in the infected ear. Similar to challenge with Der p 2, WASp KO mice infected with *L. major* showed an altered immune response with increased expansion of IFNγ-producing CD8$^+$T cells in the draining lymph node.

Finally, we provide an explanation for increased number and activation of CD8$^+$ T cells in WASp deficiency by showing that WASp KO DCs had increased capacity to cross-present exogenous antigen and activate CD8$^+$ T cells.
Conclusions: How can immunodeficiency be associated with increased immune cell activation to seemingly harmless substances such as allergens? Our findings reveal that even though WASp deficiency leads to a compromised immune response, WASp KO cells can respond to allergens and parasite infiltration in the skin. However, skin inflammation in WASp KO mice causes a skewed immune response with DC mediated CD8$^+$ T cell responses at incorrect sites.

P0936
Substrates of the MHC-I antigenic pool derives from DRiPs rather than from mature protein

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Purpose/Objective: Defective ribosomal products (DRiPs) are a subset of newly synthesized proteins which are rapidly degraded by the proteasome. The observation that antigen presentation on MHC class I is detectable immediately after antigen expression led to the conclusion that peptides presented on MHC class I molecules mostly derive from DRiPs. However it still remains unclear whether and to what extent mature proteins are used as antigenic substrates.
Materials and methods: In our study we designed a conditional antigen expression system that enables to investigate antigen presentation from mature proteins by inducing their rapid proteasomal degradation in the absence of ongoing antigen synthesis.
Results: Target cells in which expression of two Epstein-Barr virus antigens was induced were rapidly recognized by antigen-specific CD8+ T cells in a time and dosage dependent manner, demonstrating that antigen presentation was linked to antigen synthesis. As opposed to this finding, T cell recognition of antigen presenting cells that contained large amounts of mature protein was not detectable even after rapid proteasomal degradation.
Conclusions: Based on these results we could demonstrate that protein synthesis is essential for MHC-I antigen presentation. More importantly we could exclude that mature proteins enter the antigenic pool for MHC-I antigen presentation. These results have implications

for the design of immunotherapeutic strategies that aim at targeting proteins with increased half-lives and hence overexpressed in tumors.

P0938
The cold-shock protein YB-1 controls T-cell proliferation and survival

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Purpose/Objective: The cold-shock protein YB-1 is an oncogene highly expressed in tumor cells and associated with increased cell survival, proliferation and migration. Its localization within the nucleus and enhanced expression of mRNA has been shown to correlate with poor prognosis for tumor patients. Although it has a central role in tumor cells such as breast, ovarian, and lung cancer, its role in T cell responses has not been appreciated, yet.
Materials and methods: $CD4^+$ T cells isolated from PBMCs or Jurkat T cells were stimulated either with anti-CD3 and isotype or with anti-CD3 and anti-CD28 coupled sulfate latex beads at the ratio 1:1 and subcellular fractions were analyzed by Western blot, expression of mRNA by real-time PCR, and expression of intracellular molecules and surface receptors by FACS analysis. YB-1 was manipulated by lentiviral transduction of YB-1shRNA.
Results: YB-1 protein was unambiguously expressed in naïve and effector/memory human $CD4^+$ T cells. In contrast to $CD4^+$ T cells, the T-ALL cell line Jurkat showed an enhanced localization of YB-1 in the nucleus independently of stimulation. To evaluate YB-1 mRNA expression in T cells as a diagnostic marker for unintentional T cell responses, stimulated $CD4^+$ T cells were analyzed under inflammatory conditions using IFN-γ and TGF-β. Expression of YB-1 mRNA revealed rather decreased expression of YB-1 mRNA in stimulated cells in the presence ofcytokines. Quantification of YB-1 mRNA expression is not suited as diagnostic marker for excessive T cell responses, as shown for tumor growth. However, shRNA-mediated knockdown of YB-1 resulted in abrogated proliferation and increased apoptosis of $CD4^+$ T cells and Jurkat cells that was rescued by IL-2 administration. Upon stimulation of human $CD4^+$ T cells with anti-CD3 and anti-CD28, YB-1 protein was highly expressed in the cytoplasm and the nucleus of T cells starting 16 h after initiating stimulation. The accumulation of YB-1 within the nucleus was inhibited in stimulated $CD4^+$ T cells when RSK1/2 was inactivated with a specific inhibitor and to a lesser extent when Akt and PKCa were inactivated thus, identifying the MEK-ERK-RSK signalling pathway in YB-1 S^{102} phosphorylation in T cells as a prerequisite for YB-1 translocation in the nucleus.
Conclusions: Altogether, our data demonstrate that YB-1 is tightly controlled in T cells and is centrally involved in T cell proliferation.

P0939
The Crucial Role of ICOS Driving the Onset of Lupus in Sle1 Mouse Model

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Purpose/Objective: The pathophysiological hallmark of lupus is the production of antibodies (Abs) against self-antigens. Studies in both human and animal models have reported increased expression of Inducible T cell Costimulator (ICOS) on T follicular helper (T_{FH}) cells in several autoimmune conditions, including lupus. T_{FH} cells are critical players in the formation of germinal centers (GCs) and under pathological condition can contribute to skewing the differentiation of B cells into hyperactive germinal center (GC) B cells and autoAbs producing plasma cells (PCs). Increased numbers of T_{FH}-like cells have been associated with high levels of autoAbs and have correlated with disease activity in patients with active lupus. Furthermore, several autoimmune animal models, including B6.Sle1 mice have suggested that abnormalities in T_{FH} cell homeostasis may represent one of the mechanisms of breaking tolerance resulting in the production of autoAbs.

The aim of this study is to explore the role of ICOS in the differentiation of T_{FH} cells on B6.Sle1 mice and thus decipher the function of T_{FH} cells during the onset of murine lupus.
Materials and methods: B6.Sle1 were crossed with B6.$ICOS^{-/-}$ animals to generate B6.Sle1 × $ICOS^{-/-}$ mice. Secondary lymphoid tissues and peripheral blood from B6.Sle1 × $ICOS^{-/-}$, B6.Sle1, B6.$ICOS^{-/-}$ and C57Bl/6 (B6) mice were collected for phenotypic analysis of T and B lymphocytes along with serum immunoglobulins (Igs) and autoAbs analysis.
Results: Immunophenotypic analysis revealed that *Icos* deficiency in B6.Sle1 mice restored the ratio of naïve/memory of CD4 T cells compared to B6.Sle1 animals. Moreover, lack of ICOS in B6.Sle1 mice statistically influenced the numbers of T_{FH} cells, which affected the numbers of GC B cells and PCs. Importantly, these changes diminished significantly the levels of Igs and autoAbs [ANA, double strand (ds) DNA and histone] in B6.Sle1 × $ICOS^{-/-}$ mice as compared to B6.Sle1 animals. Finally, while notably expanded in B6.Sle1, the numbers of T regulatory $Foxp3^+$ cells in B6.Sle1 × $ICOS^{-/-}$ animals returned to levels comparable to B6 mice.
Conclusions: Our results suggest that ICOS regulates the homeostasis of T_{FH} cells and influences the function of T_{FH} during GC B cell responses. Therefore, therapies targeting the ICOS signaling pathway may offer new opportunities to reduce disease activity in patients with lupus.

P0940
The immunopathogenesis of ankylosing spondylitis: a dendritic cell perspective

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Purpose/Objective: Ankylosing Spondylitis (AS), a member of the Spondyloarthritis family, is a chronic systemic inflammatory disease primarily affecting the axial skeleton. Despite the fact that 95% of AS patients express the MHC Class I molecule HLA-B27, the pathogenic role of this molecule remains elusive. Dendritic cells (DCs) are essential for directing and inducing immune responses. Studies in our laboratory using HLA-B27 transgenic (TG) rats, an animal model of spondyloarthropathy, have uncovered deficiencies in DC populations. We hypothesised, therefore, that DC populations in AS patients would be altered, contributing to chronic inflammation. We set out to characterise and functionally compare DC populations between AS patients and healthy controls, aiming to understand the role of DCs in AS pathogenesis.
Materials and methods: AS patient peripheral blood samples were obtained from the AS clinic at Glasgow Royal Infirmary. Healthy donors were recruited from Glasgow University. Peripheral blood mononuclear cells (PBMCs) were isolated using a ficoll gradient and analysed for T cells and DC subsets using 7-colour flow cytometry. Flow sorted DCs were co-cultured with allogeneic T cells, where T cell proliferation was measured by CFSE dilution and chemokine expres-

sion was analysed by flow cytometry. Circulating plasma cytokines were measured by Luminex.
Results: Our initial experiments have successfully identified all known blood DC subsets in both healthy controls and AS patients. AS patients have an increased proportion of blood $CD16^+$ $CD11c^+$ DCs, in addition to an increased proportion of circulating $CD4^+$ $CCR6^+$ activated T cells. Using mixed lymphocyte reactions, we observe a tendency for AS $CD16^+$ DCs to induce greater levels of CCR6 on responding naïve T cells compared to those isolated from healthy controls. In addition, this subset preferentially induced secretion of a variety of pro-inflammatory cytokines including TNFα from these co-cultures.
Conclusions: Our results demonstrate differences in the proportions of several DC subsets, between AS patients and healthy controls. Specifically, in AS patients the pro-inflammatory $CD16^+$ $CD11c^+$ DC subset is expanded, and this subset induces a larger proportion of naïve T cells to express CCR6. These data, combined with the observed increase in the proportion of $CCR6^+$ $CD4^+$ activated T cells in AS patient blood may suggest a pathogenic role for these DCs.

P0942
The proteasome system in infection: Impact of ß5 and LMP7 on composition, maturation and quantity of active proteasome complexes

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Purpose/Objective: Proteasomes are involved in MHC class I antigen presentation, inflammation and defense against oxidative stress. Mammals express two sets of catalytic proteasome subunits: the constitutive subunits $\beta1$, $\beta2$ and $\beta5$ and the immunosubunits LMP2, MECL-1 and LMP7. According to the rules of cooperative proteasome assembly, the LMP7-propeptide (proLMP7) is required for maturation of LMP2/MECL-1-containing precursors to immunoproteasomes, while the $\beta5$-propeptide (pro$\beta5$) promotes integration into $\beta1/\beta2$-containing precursors, resulting in the formation of constitutive proteasomes. However, these rules have been defined in steady state cell lines and thus we raised the question, whether they are also valid under inflammatory conditions. Further, it has been described that LMP7 accelerates the rate of proteasome assembly and we address, how this function of LMP7 affects the proteasome composition, maturation and quantity in infection.
Materials and methods: To analyse the function of proLMP7 and pro$\beta5$ under inflammatory conditions, $lmp7^{-/-}$ MEFs were reconstituted with full length LMP7 or $\beta5$ or chimeric subunits, in which proLMP7 was fused to $\beta5$ (proLMP7m$\beta5$) or pro$\beta5$ was fused to LMP7 (pro$\beta5$ mLMP7). The integration of the full-length or chimeric subunits into proteasomes was analysed by co-immunoprecipitation in IFNγ-stimulated $lmp7^{-/-}$ MEFs. The impact of LMP7 on proteasome composition, maturation and quantity in infection, was determined in $lmp7^{-/-}$ and wild-type mice infected with *Listeria monocytogenes*..
Results: We found that pro$\beta5$ mediates predominant integration into LMP2/MECL-1-containing precursors in IFNγ-stimulated $lmp7^{-/-}$ MEFs and infected $lmp7^{-/-}$ mice. Further, we identified the propeptides as the critical factors determining the efficiency of proteasome maturation, with proLMP7 displaying a higher chaperone activity as compared to pro$\beta5$. In infection, induction of LMP7 not only results in rapid formation of immunoproteasomes, but also increases the proteasome quantity within the infected tissue.
Conclusions: Since $\beta5$ mediates predominant integration into mixed LMP2/MECL-1/$\beta5$ proteasomes in IFNγ-stimulated cells and infected mice, the rules of cooperative proteasome assembly do not appear to be valid under inflammatory conditions. Moreover, we identify a LMP7-dependent mechnism, which increases the proteasome quantity in infected tissues and is based on the high chaperone activity of proLMP7.

P0943
The protective role of interferon gamma in EAE, is not due to effects on dendritic cells

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Purpose/Objective: Interferon gamma has been shown to play an essential role in the immune response towards intracellular pathogens. However recent publications point to an additional, regulatory function, as mice deficient for either IFNγ or the receptor are highly susceptible for induction of EAE and show a severe outcome of the disease. We analyzed the role of IFNγ signaling in dendritic cells (DCs) in EAE by using a conditional receptor knock-out model.
Materials and methods: We crossed mice with floxed *Ifngr2* to CD11c-Cre mice (CD11c/IFNγR2 KO). EAE was induced by immunization with MOG_{35-55} in emulsion with CFA and mice were analyzed for effector and regulatory T cells, as well as for expression of CD80, CD86 and PDL-1/-2 on DCs. Additionally, we generated BMDCs and analyzed their capability for *in vitro* T cell differentiation and expansion.
Results: We found, that loss of IFNγ signaling in DCs, led to a slightly more severe outcome of EAE, but did not enhance the infiltration of effector T cells in the CNS and did not alter the number of regulatory T cells in the periphery. Culture of LPS and IFNγ stimulated and MOG_{35-55} pulsed control BMDCs with $CD4^+$ T cells from 2D2 mice, led to significantly more $FoxP3^+$ T cells, which were additionally more expanded.
Conclusions: Our results suggest that the protective effect of IFNγ in EAE is not due to effects on DCs, as we could not see a difference in the number of effector T cells or the expression of stimulatory/inhibitory molecules on DCs. However, our *in vitro* data propose a role of IFNγ signaling in the generation and expansion of regulatory T cells.

P0945
The role of lysosomal trafficking regulator LYST in antigen presentation

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Purpose/Objective: Chediak -Higashi syndrome (CHS) is a genetic disorder caused by mutations in the lysosomal trafficking gene, LYST. Patients with this disorder suffer from partial albinism, excessive bleeding, and recurrent bacterial infections. The murine equivalent of CHS, *beige* mice exhibits a similar phenotype. A prominent feature of this disease is the accumulation of enlarged lysosome-related granules in a variety of cells. In order to generate appropriate $CD8^+$ T cell-mediated immune responses to viral, bacterial, or tumor associated protein antigens, professional antigen-presenting cells acquire these antigens from the extracellular milieu;

process them at the cell surface. This process of 'cross-presentation'is known to occur most efficintly in dendritic cells (DCs). However, the underlying mechanistic and molecular details have remained largely unclear. Here we investigated the role of LYST in the regulation of dendritic cell function, particularly in the process of antigen cross-presentation.

Materials and methods: Utilizing a genetic system of LYST-deficient mice our study followed a multidisciplinary approach, which involved bio-chemical, cell biological and immunological methods to analyse the impact of lysosomal trafficking regulator LYST on antigen presentation and other functional aspects of dendritic cells.

Results: Our studies revealed that LYST is critically involved in cross-presentation in DCs. LYST MUTANT DCs from *beige* mice showed a significantly impaired ability to present ovalbumin (OVA)-peptide on the cell surface upon uptake of OVA-protein coupled beads. As a consequence, LYST mutant DCS only poorly activate OT-1CD8^{+} T cells which specifically recognize OVA-peptide presented by MHC-1molecules. The observation that the uptake of apoptotic cells through phagocytic processes appears to be intact in LYST mutant DCs suggests that the impaired ability to present antigen on the surface likely is due to defects in antigen processing at endosomal compartments.

Conclusions: Our studies reveal a so far unrecognized function of LYST as a novel effector molecule in antigen cross -presentation in dendritic cells.

P0948
Vav1 regulates transcription and transport of MHCII molecules

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Purpose/Objective: Vav1 is a guanine nucleotide exchange factor (GEF) for Rho GTPases which is exclusively expressed in cells of the hematopoietic system. In addition to its well-documented GEF-activity it was suggested to have other functions, due to the presence of multiple domains and nuclear localization signals in its protein structure. Although GEF-dependent and GEF-independent functions of vav1 have been implicated in T cell development and T cell receptor signaling, the different roles of vav1 in antigen-presenting cells (APC) are poorly understood.

Materials and methods: To evaluate differential phenotypes and functions of APC from vav1$^{-/-}$ mice in comparison to WT mice, we performed experiments with resting and stimulated primary B cells, dendritic cells (DC) and macrophages as well as bone marrow-derived (BM) DC and macrophages.

Results: We found that vav1 is an important regulator of MHCII expression and transport. Microarray analysis of un-stimulated BM-macrophages revealed a role of vav1 in the transcription of the MHCII locus, which was not based on changes in CIITA expression. Primary immune cells from vav1-deficient mice as well as BM-APC had a significantly lower constitutive and inducible surface expression of MHCII molecules resulting in a diminished capacity for T cell activation. Using 6-thio-GTP, a specific inhibitor of vav1-GEF function, we were able to show that the GEF-activity is required for MHCII up-regulation after stimulation with LPS and IL-4/anti-CD40, respectively, but were dispensable for basal MHCII expression in un-stimulated cells. Furthermore, our data show that vav1 does not only control transcription of the MHCII locus, but is also an important regulator of MHCII protein transport to the cell surface.

Conclusions: Collectively, we show that vav1 regulates transcription and intracellular transport of MHCII molecules in antigen-presenting cells and indicates that inhibition of vav1 might be useful for the therapy of inflammatory diseases.

Poster Session: Fundamental Inflammatory Processes

P0949

A potential role for the X chromosome in the gender differences observed in the innate immune response

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Purpose/Objective: Gender influences the severity and evolution of various inflammatory conditions. Many studies have demonstrated the important role of sex hormones in modulating the inflammatory response. However, recent clinical studies showed gender differences in inflammatory markers of prepubertal children, suggesting a genetic contribution.

Materials and methods: We determined some leukocyte parameters depending on X-linked genes in 24 healthy adults of both sexes and 8 patients with sex chromosome aneuploidy: 4 patients with Klinefelter syndrome (XXY) and 4 patients carrying an extra Y chromosome (XYY). We measured neutrophil respiratory burst by chemiluminescence, leukocyte expression of CD11b and CD99 and cytokine production (IL-1β, IL-6, IL-8, IL-10, IFN-γ and TNF-α) in whole blood.

Results: The neutrophil respiratory burst was not different between groups ($P > 0.05$) although the main NADPH oxydase subunit gp91phox is linked to the X chromosome. Median CD99 monocyte and lymphocyte expressions were higher in patients with sex chromosome aneuploidy compared to healthy adults in the basal state as after stimulation with fMLP and LPS ($P < 0.05$). This indicates a sex chromosome dose effect on CD99 expression. CD99 plays a major role in the migration of leukocytes through endothelial junctions. Its gene is located on the pseudoautosomal region PAR1, close to the boundary with X-linked regions where it can accidentally be silenced in the inactivated X chromosome of female cells. Additionally, men with Klinefelter syndrome (XXY) produced significantly less TNF-α compared to healthy men (XY) ($P < 0.01$) and had a tendency to produce less IL-6 ($P = 0.07$) in response to LPS. We did not observe any difference in the levels of 17β-estradiol and testosterone between these two groups ($P > 0.05$) suggesting an influence of the X chromosome numbers rather than the sex steroids levels.

Conclusions: Although performed in a small number of patients, our study highlighted important differences in men carrying an extra X chromosome in terms of the innate immune response to endotoxin through expression of a diapedesis marker and secretion of inflammatory cytokines depending on the TLR activation. These differences suggest a role for the X chromosome in the sex-specific response to acute inflammatory challenge, independently of hormonal impregnation.

P950

ABR-215757, an immunomodulatory compound, reduces the accumulation of cells during acute peritonitis

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Purpose/Objective: The quinoline-3-carboxamides (Q compounds) are a group of chemical immunomodulators that have shown efficacy in several mouse models of human autoimmune disease. Currently, one such compound, ABR-215757 (5757), is in clinical trial for scleroderma. However, the mechanism behind the immunomodulating capacity of the Q compounds is largely unknown. The aim of this study was to evaluate the effect of 5757 on cell recruitment, as this is one potential mechanism for the Q compounds.

Materials and methods: For this purpose, we used a mouse model of acute peritonitis induced either by intraperitoneal injection of heat-shocked necrotic cells or Imject alum. Also, monocyte recruitment was induced by the injection of monocyte chemoattractant protein-1 (MCP-1).

Results: Upon induction of peritonitis using heat-shocked necrotic cells, treatment with 5757 reduced the number of cells accumulated in the peritoneum. This was mainly due to a reduction in the number of inflammatory monocytes, but also neutrophils and eosinophils. Similar results were obtained during the early phase of inflammation induced by immunization with Imject alum. Also, the recruitment of inflammatory monocytes to the omentum, by intraperitoneal injection of MCP-1, was abolished by treatment with 5757. Seven-day treatment with 5757 during steady state conditions similarly reduced the influx of inflammatory monocytes, neutrophils and eosinophils to the omentum as well as peritoneum, suggesting an effect on the homeostatic migration of cells as well.

Conclusions: Our results indicate that 5757 reduces the accumulation of cells in the peritoneum during acute inflammation, as well as during steady state. Future studies include performing transmigration assays using Boyden chambers, allowing monocytes to migrate across either a resting or activated endothelial cell layer pretreated with 5757. This will help us understand if the effects of 5757 observed in this study are due to an impact on endothelial cells and if cells in the blood stream, as a consequence, are unable to migrate across the endothelial cells of the blood vessels. The information obtained in this study could help us clarify the mechanism of the Q compounds in relation to cell recruitment during autoimmune disease.

P951

Altered distribution of neutrophils and NK cells in collagenase-induced osteoarthritis

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Purpose/Objective: Osteoarthritis (OA) is a degenerative joint disease associated with high disability and pain. The aim of the present study is to evaluate the distribution and trafficking of neutrophils and NK cells in an experimental model of osteoarthritis.

Materials and methods: SCID mice received an intra-articular (i.a) injection of collagenase at the knee joint (CIOA mice). In order to compare the progression of OA with an acute inflammatory process, a group of SCID mice were i.a. injected with zymosan (ZIA mice). The degree of joint damage were determined after H&E and toluidine blue staining. The distribution of neutrophils and NK cells and the expression of surface CXCR2, 4 and galectin-9 were evaluated in lymphoid organs and bodily fluids by flow cytometry at day 7 of the disease model.

Results: Two populations of neutrophils, LY6G^{high} and LY6G^{low} were found in bone marrow (BM) and spleen. While BM LY6G^{high} cells expressed CXCR 2 and galectin-9, LY6G^{low} cells were negative for CXCR 2 and 4 but positive for galectin-9. Down-regulated expression of CXCR 2, 4 and galectin-9 on LY6G^{high} cells at day 7 of CIOA may correlate with the mobilization of these cells from the BM. CIOA NKp46 cells expressed CXCR4 and lost the surface CXCR2 in BM but

in a lesser extent than ZIA population. In spleen, CXCR2 and 4 decreased on CIOA LY6G^{high} cells similarly to ZIA cells. LY6G^{low} population negative for CXCR2, 4 up-regulated galectin-9 only in CIOA mice suggesting its specific role in the osteoarthritic process. CIOA NKp46 cells in spleen down-regulated surface CXCR2 and 4. The percentages of CXCR 4+ cells in blood were strongly reduced in CIOA (for NK cells) and ZIA (for NK cells and neutrophils) groups suggesting that the cells with decreased expression of CXCR 4 can enter the circulation. A lot of the cells in ZIA synovial fluid were CXCR2+ and had down-modulated CXCR4 while CIOA phenotype was more similar to control.

Conclusions: Our investigations shed a new light on the mechanism of LY6G neutrophils and NK cell trafficking in OA. Different modulations observed on these populations, suggest that the exploration of these mechanisms in OA could lead to the development of novel therapeutic strategies for the disease.

P953

CCR7 deficiency causes manifestation of spontaneous autoimmune gastritis and chronic diarrhea in mice

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Purpose/Objective: The chemokine receptor CCR7 is a central regulator in the maintenance of cellular homeostasis of mucosal tissues. CCR7 deficiency in mice leads to massive accumulation of lymphocytes within the gastric mucosa along with the development of tertiary lymphoid organs (TLOs) and histomorphological changes including cystic hyperplasia. We investigated if gastrointestinal aberrant lymphoid aggregates in CCR7-deficient mice were functionally linked with the development of autoimmune gastritis (AIG) and intestinal inflammation.

Materials and methods: Gastrointestinal pathogenesis was monitored using immunohistochemistry, flow cytometry analysis, and ELISA. Sonic hedgehog, H^+K^+-ATPase alpha, cystic fibrosis transmembrane conductance regulator (CFTR), and epithelium sodium channel (ENAC) expression was quantified by quantitative PCR or by immunoblot analysis. Colonic chloride secretion and electronic sodium transport was analysed in Ussing chambers.

Results: CCR7-deficient mice developed an early AIG accompanied by metaplasia of the gastric mucosa and the formation of gastric TLOs. Moreover, we found that CCR7-deficient mice displayed enhanced formation of intestinal lymphoid follicles in the colon and frequently suffered from chronic diarrhea and the development of severe anorectal prolapse. We found an elevated proportion of activated T cells within the gastric and colonic mucosa and an increased local production of the cytokines IL-1beta and IL-17. The induction of a defined inflammatory microenvironment in the stomach led to increased gastric pH via suppression of the gastric morphogenic factor sonic hedgehog. In the colon, IL-1beta production reduced ion channel-mediated sodium absorption and cloride secretion in colonocytes which led to altered net ion flux and a Na^+-malabsorptive-type of diarrhea.

Conclusions: In conclusion, CCR7-deficient mice reveal an immune-mediated condition which causes a dosed immune response of pro-inflammatory cytokines resulting in the development of autoimmune gastritis and chronic diarrhea.

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P0954

Effects of fibroblast co-culture on the response of immature porcine cartilage explant tissue to erythropoietin and interleukin-1 beta in explant models of arthritic disease

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Purpose/Objective: To characterise the responsiveness of immature porcine nasal explant tissue to the inflammatory mediator interleukin-1 beta (IL-1β) and the human recombinant cytokine hormone erythropoietin (rHuEPO) in the absence and presence of fibroblast cells.

Materials and methods: Explant discs from 1 to 2 month old piglets were pre-cultured for 24 h in the absence or presence of 3T3 fibroblasts, prior to addition of optimum concentrations of rHuEPO (25 μU/ml), either alone or in combination with IL-1β (20 ng/ml). Media and treatments were replenished every 24 h and supernatants were tested for nitric oxide (NO) release and glycosaminoglycan (GAG) breakdown.

Results: The effects of rHuEPO on explant tissue were comparable to that of IL-1β treatment, and were greater in combined rHuEPO/IL-1β treatments than in individual treatments; fibroblast co-cultures significantly further increased NO and GAG release. Interestingly, while the nitric oxide synthase (NOS) inhibitor NG-nitro-L-arginine methyl ester hydrochloride (L-NAME) reduced NO and GAG release to baseline levels in controls lacking fibroblasts, the NOS inhibitor had no effect on fibroblast co-cultures.

Conclusions: The results confirm earlier studies, which showed that independent of its established erythropoietic role, pleiotropic rHuEPO can induce NO and GAG release in immature cartilaginous explant tissue to a level similar to that observed with IL-1β treatment. The amplifying effect of fibroblast co-cultures implies a synergistic interaction between immature explant cells and fibroblasts in the stimulation of GAG breakdown and NO release, possibly mimicking the role of pannus tissue on cartilage tissue breakdown in rheumatoid arthritis. Further support for a direct role of rHuEPO in matrix remodelling comes from preliminary experiments, which have confirmed that the mRNA for EPO receptor is expressed in 3T3 fibroblasts and that changes in MMP-1 and MMP-13 expression appear to occur in a dose-responsive manner in primary cultures of human synoviocyte-like fibroblasts, following rHuEPO and/or IL-1β treatment. Collectively, these results support the contention that immature fibroblast co-cultures are a suitable model for the study of erythropoietin in the pathogenesis of arthritic diseases.

P0956

High MMP-2, MMP-9 and low TIMP-1 expression in the spinal cord of rats correlate to CNS autoimmunity resistance

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Purpose/Objective: Experimental autoimmune encephalomyelitis (EAE) is a model of multiple sclerosis (MS), an inflammatory, demyelinating and neurodegenerative disease of the CNS. We have previously shown that Dark Agouti (DA) rats immunized with spinal cord homogenate (SCH) and Complete Freund's adjuvant (CFA) develop

severe form of EAE, while Albino Oxford (AO) rats remain fairly unaffected. Although initial infiltrates of immune cells were detected within the CNS of both strains, they multiplied in DA rats during the course of EAE, but remained low in AO rats. The restricted infiltration in AO rats imply that regulatory mechanisms within the CNS contribute to their resistance to EAE. Among various molecules within the CNS, matrix metaloproteinases (MMP) play significant role in initiation, progression and resolution of inflammation in MS and EAE. Our aim was to investigate gene expression of MMP-2 and MMP-9 and their inhibitor TIMP-1 in AO and DA rats CNS during the course of EAE.

Materials and methods: EAE was induced in rats by immunization with SCH + CFA. Spinal cords of both strains were isolated from untreated rats and from immunized rats at the time of onset, peak and recovery of EAE in DA rats. SC immune cells (SCIC) were isolated using Percoll gradient (30%/70%). Gene expression in SCH and SCIC was measured by real-time RT-PCR.

Results: Immunized, DA rats, but not AO rats, developed clinically manifested EAE. The expression of the examined genes in untreated rats was low in both strains. After immunization, MMP-9 and TIMP-1 gene expression was increased in SCH of both strains, while MMP-2 was increased only in AO rats. MMP-2 and MMP-9 gene expression was higher and TIMP-1 gene expression was lower in AO rats than in DA rats SCH and SCIC.

Conclusions: Resistance of AO rats to EAE correlated to high gene expression of MMP-2 and MMP-9 and low expression of TIMP-1. Such a pattern of MMP-2, MMP-9 and TIMP-1 expression imply that these molecules might have additional roles in EAE besides previously described promotion of neuroinflammation.

P0957

Innate neutrophil function is regulated locally through IL-6 control of prostaglandins

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Purpose/Objective: Interleukin-6 (IL-6) is vital for competent antimicrobial host defence. Mice deficient in IL-6 are unable to control bacterial peritoneal infections and show increase dissemination and reduced bacterial clearance. Although the mechanism underlying this response is currently unknown, we propose that this is linked to the ability of IL-6 to govern the pattern of leukocyte recruitment that directs transition from neutrophils to monocytic cells.

Materials and methods: Real time quantitative PCR of human peritoneal mesothelial cells (HPMC) and mouse peritoneal membrane was used to examine IL-6 mediated expression of enzyme systems implicated in antimicrobial host defence. A Peritonitis model of *Staphylococcus epidermidis* (*S. epi*) assessed the host response to infection in wild type, IL-$6^{-/-}$ and IL-$6R^{-/-}$ mice. Flow cytometry characterised neutrophil effector function in response to live bacteria. Bioactive lipids and inflammatory cytokines in peritoneal lavage and tissue culture supernatants were quantified by mass spectrometry and ELISA.

Results: Bacterial clearance within the IL-6 deficient mouse is compromised; the time frame in which this occurs suggests a defective innate component. Although the antibacterial effector characteristics of patrolling neutrophils from IL-6 deficient mice was not compromised, once recruited to the peritoneal cavity following bacterial challenge, these neutrophils show impaired respiratory burst and phagocytic activity. Analysis of inflammation related genes in HPMC showed an IL-6 dependent COX2 activation, suggesting a role for PGE2 in the IL-6 control of acute infection. PGE2 (1 μM) treatment significantly reduced mouse neutrophil respiratory burst and bacterial phagocytosis when stimulated with live *S. epi*. IL-6 deficient mice show poor control of membrane derived COX2 and increased levels of at baseline compared to wild type mice.

Conclusions: These studies indicate that IL-6 control of non-haemopoietic stromal cells is important for regulating the innate effector characteristics of infiltrating neutrophils. In this regard, IL-6 control of COX2 may govern this response, with the production of COX2 derived PGE2 (and other COX2 related lipids), impacting the innate response to bacterial challenge.

P0958

Lesional IL-21 expression correlates with functional germinal center formation and T follicular helper cell infiltration in the salivary glands of Sjögren's syndrome

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Purpose/Objective: IL-21 is a pro-inflammatory cytokine that plays a key role in the activation and differentiation of B cells. B cells infiltrate the salivary glands (SG) of Sjögren's syndrome (SS), an autoimmune disease characterized by immune infiltration in the lacrimal and salivary glands leading to exocrine dysfunction. In SS SG, B cells are organized as functional ectopic germinal centers (GC) and are pivotal in SS pathogenesis. Follicular helper T cells (Tfh) abundantly express IL-21 and contributes to GC B cell affinity maturation via induction of AID, somatic hypermutation and class switching. We aimed to investigate IL-21 and TFh expression in SS SG and their relationship with the development of functional ectopic GC.

Materials and methods: IL-21 and IL-21R mRNA expression was assessed by Taqman PCR in 22 labial SG of patients with SS and 17 with non-specific chronic sialadenitis (NSCS). Expression levels were correlated with genes regulating ectopic GC formation such as CXCL13 and Ltb and B cell function such as BAFF, AID, Pax5 and Blimp1. In addition, GC formation in the SG was assessed by IHC for B/T cell segregation and follicular dendritic cell (FDC) networks. Finally, Tfh cells infiltration in the SG was assessed by IF for CD3/CD45RO/ICOS and PD1.

Results: SG of SS patients displayed higher expression of IL-21 and IL-21R mRNA compared to NSCS (mean fold increase ± SEM 38 ± 12 versus 5 ± 4, $P = 0.02$ for IL-21 and 59 ± 13 versus 12 ± 2, $P = 0.01$). In SS, IL-21 mRNA strictly correlated with the levels of CXCL13 (Spearman's $r = 0.697$, $P < 0.0001$) and Ltb ($r = 0.478$, $P < 0.001$) which were closely associated with the formation of CD21L+ ectopic GC. Furthermore, IL-21 expression was associated with functional B cell activation as shown by its correlation with AID ($r = 0.540$, $P < 0.0001$) and Pax5 ($r = 0.456$, $P < 0.002$). Finally, a subset of CD45RO/ICOS/PD1+ T cells highly resembling Tfh were invariably observed in the presence of ectopic GC characterised by FDC networks but not in FDC- or NSCS SG.

Conclusions: Here we show that IL-21 expression is significantly increased in the SG of SS patients and strongly correlate with ectopic GC formation, functional B cell activation and accumulation of Tfh. These data strongly implicate IL-21 and Tfh cells in the development and maintenance of functional ectopic GC in the SG and could represent novel therapeutic targets in SS.

P0959

Leukocyte associated Ig-like receptor-1 is a novel receptor for the immunomodulatory surfactant protein D

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Purpose/Objective: The collagenous C-type lectin surfactant protein D (SP-D) is an immunomodulatory multi-trimeric protein. Its collagenous triple helices are linked via its neck domains to distinct carbohydrate recognition domains (CRD). Most putative SP-D receptors interact with the CRDs of SP-D. Leukocyte Associated Ig-like Receptor-1 (LAIR-1) is an inhibitory immune receptor that binds collagen. We hypothesize that the immunomodulatory functions of SP-D can be mediated via binding to LAIR-1.
Materials and methods: We investigated binding of SP-D to LAIR-1 by using a cell-free, protein-based system and with surface plasmon resonance experiments. By using mannan-coated plates, we determined whether LAIR-2 binding to SP-D interferes with the sugar-binding properties of SP-D. In addition, we used LAIR-1 expressing reporter cells to investigate the capacity of SP-D to trigger the receptor.
Results: We showed binding of SP-D to LAIR-1 and its family member LAIR-2. Furthermore, we determined that this binding was mediated via the collagen domain of SP-D. Binding of SP-D to LAIR-2 did not alter the binding of SP-D to mannan, indicating that the opsonin function of SP-D was not affected by binding to LAIR. Binding of SP-D to LAIR-1 expressed on cells triggered the receptor, thereby showing the functionality of this interaction and adding further evidence for a potential functional role of SP-D/LAIR-1 interactions.
Conclusions: We demonstrate that SP-D is a functional ligand for the immune inhibitory receptor LAIR-1. Thus, we have identified a novel pathway for immunomodulatory functions of SP-D.

P0961

Neutrophils are the key initiators of early osteoclastogenic events in experimental arthritis model

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Purpose/Objective: The potential of neutrophils to take part in inflammatory events in arthritis were recently highlighted. Herein we studied the neutrophils ability to promote destruction and bone resorption. We evaluated the RANKL expression, IL-17 and IFNγ production of peripheral and synovial neutrophils isolated from mice with zymosan-induced arthritis (ZIA).
Materials and methods: BALB/c mice were injected i.a. with zymosan. The organ distribution of neutrophils was evaluated by flow cytometry at day 7 of disease. Peripheral and synovial neutrophils were purified by Percoll gradient. Surface RANKL expression and intracellular production of IL-17 and IFNγ were determined by flow cytometry. Peripheral neutrophils were co-cultured with osteoclast bone morrow precursors and the number of mature osteoclasts was evaluated after TRAP staining. In a set of experiments we eliminated neutrophil population in SCID mice by i.p. administration of etoposide for 3 days prior zymosan injection.
Results: We found elevated number of Ly6G/CD11b cells in synovial fluid, blood and bone morrow of ZIA mice. Arthritic peripheral neutrophils showed up-regulated RANKL expression and increased IL-17 production in comparison to naïve mice. This ability of neutrophis was additionaly amplified *in vitro* by exogenous IL-17. In ZIA mice most of the synovial neutrophils were double IL-17/IFNγ producers. The elimination of neutrophils in periphery by etopiside resulted in a significantly decreased IL-17 synovial levels and joint destruction. *In vitro* experiments demonstrated that neutrophils promoted IL-17-induced osteoclastogenesis.
Conclusions: We propose that neutrophils receive signals for emerging inflammatory process in the bone morrow. Migrating to the periphery they acquire activated phenotype, express RANKL and IL-17 and thus contribute to early destructive events in arthritic synovium. Our results open new perspective for design of novel treatment strategy to inhibit abnormal bone destruction in arthritis.

P0962

NOD2/CARD15, IL-17A and IL-23R gene polymorphisms in sarcoidosis

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Purpose/Objective: Sarcoidosis is a multiorgan granulomatous disease with clinically apparent inflammatory lesions of different activity. Our former results highlight the influence of HLA-associated factors on the extent of inflammation. In the present study we focused on the polymorphisms within the NOD2/CARD15 (SNP8: rs2066844, 2104C>T; SNP12: rs2066845, 2722G>C; SNP13: rs2066847, insC), IL-17A (rs2275913, 197G>A) and IL-23R (rs11209026, 1142G>A) encoding genes, which products promote Th17 response.
Materials and methods: Two hundred and thirty one individuals were studied, including 48 Polish patients with sarcoidosis, 13 of which presented with Löfgren's syndrome (LS) and 83 control subjects. *NOD2/CARD15* genotyping was performed by PCR-RFLP technique. Real-time PCR amplification with LightSNiP or TaqMan SNP Genotyping Assay was used for detection of *IL-17A* and *IL-23R* alleles, respectively.
Results: There was no significant difference in the distribution of *NOD2/CAR15, IL-17A and IL-23R* alleles and genotypes in sarcoidosis patients and controls. However, a tendency was observed towards the lower representation of *IL-23R* mutation among patients when compared to controls (1/47 versus 9/83, RR = 0.25, $P = 0.091$). LS patients more frequently presented with the *SNP13* variant (insC) when compared to healthy individuals (4/13 versus 6/80, RR = 5.43, $P = 0.031$). The prevalence of the *SNP13* variant (insC) was also seen when LS patients were compared to those lacking LS symptoms (4/13 versus 3/34, $P = 0.080$). It is postulated that the presence of any of 3 *NOD2/CARD15* SNPs influence the transcription potential of this gene. Also in the present study patients with the presence of any studied *NOD2/CARD15* mutations significantly prevailed among LS as compared to non-LS cases (7/13 versus 6/34, $P = 0.026$) and controls (7/13 versus 14/80, RR = 5.29, $P = 0.008$). In the studied group, majority of LS patients were carrying DRB1*03 (8/13 versus 8/34, $P = 0.020$) as compared with non-LS cases. Interestingly, HLA-DRB1*03-positive LS patients were more frequently carrying *NOD2/CARD15* variants (5 out of 8; 62.5%) and the concomitant presence of these 2 genetic factors was more frequent among LS as compared to non-LS patients (5/13 versus 1/34, $P = 0.004$).
Conclusions: Therefore, the contribution of the factors associated with DRB1*03 and *NOD2/CARD15* SNPs contribute to LS – highly inflammatory variant of sarcoidosis.

P0963

p110gamma PI3K deletion in LDL receptor-deficient mice reduces macrophage proliferation but not M1/M2 polarization in atherosclerotic lesions

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Purpose/Objective: Atherosclerosis is an inflammatory disease driven by atherogenic lipids that accumulate in arterial walls. It is regulated by the immune system and shares characteristics with other inflammatory diseases, such as tissue infiltration of activated monocyte/macrophages and T cells. Macrophage number in atherosclerotic lesions is controlled by monocyte migration to plaque and by *in situ* macrophage proliferation. Differentiation to an inflammatory (M1) or anti-inflammatory phenotype (M2) is also implicated in atherosclerosis progression. We studied the role of phosphoinositol-3-kinase (PI3K) p110γ in the regulation of macrophage proliferation and polarization towards pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes in atherosclerotic lesions.
Materials and methods: We analyzed atherosclerosis development in LDLR$^{-/-}$ p110$\gamma^{+/-}$ and LDLR$^{-/-}$ p110$\gamma^{-/-}$ mice, and performed expression and functional assays in tissues and primary cells from these and from p110$\gamma^{+/-}$ and p110$\gamma^{-/-}$ mice.
Results: Atherosclerotic plaques in fat-fed LDLR$^{-/-}$ p110$\gamma^{-/-}$ were smaller and had reduced immune cell infiltration compared with LDLR$^{-/-}$ p110$\gamma^{+/-}$ controls. This coincided with decreased macrophage proliferation in atherosclerotic lesions of LDLR$^{-/-}$ p110$\gamma^{-/-}$ mice. This proliferation defect was also observed in p110$\gamma^{-/-}$ bone marrow-derived macrophages (BMM) stimulated with macrophage colony-stimulating factor (M-CSF), and was associated with accumulation of the growth suppressor p27^{Kip1}. In contrast, proliferation of infiltrating T cells was unaffected in LDLR$^{-/-}$ p110$\gamma^{-/-}$ mice. p110γ deficiency did not affect macrophage polarization towards the M1 or M2 phenotypes in atherosclerotic plaques and cultured BMM.
Conclusions: Our results suggest that p27^{Kip1} accumulation and the ensuing inhibition of macrophage proliferation contribute to atheroprotection in LDLR-null mice lacking p110γ. Nonetheless, p110γ deletion does not appear to be involved in infiltrating macrophage polarization or in infiltrating T cell proliferation.

P0964

Paracrine Interactions in neuroinflammation

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Purpose/Objective: Neural progenitor cells (NPCs) are potent immunomodulatory agents, capable of promoting tissue repair in cases of neurodegenerative disease. As yet, the mechanisms by which they aid regeneration in disease conditions are unknown. Here, we investigate the interactions between NPCs and the main drivers of neuroinflammation, microglia. By studying the dynamics of a gene transcription factor, and major neuroinflammatory mediator, Nuclear Factor kappaB (NF-kB), we aim to elucidate paracrine interactions that may provide therapeutic effects in the diseased brain.
Materials and methods: We have adopted a systems level approach to study NF-kB dynamics in a mouse model of neuroinflammation. Modified murine microglial (BV.2) and NPC (C17.2) lines were used to make single cell- and dual cell-cultures, which were stimulated with different doses of a potent inflammatory agent [either tumor necrosis factor-a (TNF-a) or lipopolysaccharide (LPS)]. Population-level NF-kB activation was observed, using cells expressing NF-kB promoter-driven luciferase and live cell luminometry. This was complimented with quantitative single cell analysis of NF-kB dynamics, using cells expressing fluorescently- tagged NF-kB and confocal microscopy.
Results: Our results demonstrate interactions between microglia and NPCs in a neuroinflammatory environment. NF-kB response in these cell lines show distinct stimulus-dependant and dose-time activation profiles. Interestingly, some cells within an NPC population demonstrate NF-kB unresponsiveness to inflammatory stimuli, even at saturating doses.
Conclusions: Quantitative, multi-scale analysis of NF-kB dynamics reveals complex NF-kB-mediated interactions between microglia and NPCs. These interactions require further investigation to determine whether they serve to orchestrate a regenerative phenotype in the inflamed brain.

P0965

Required contribution of IL-6, IL-17 AND IL-23 to control the paracoccidioidomycosis

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Purpose/Objective: *Paracoccidioides brasiliensis* (Pb), a thermally dimorphic fungus, is the causative agent of paracoccidioidomycosis (PCM), one of the most frequent systemic mycosis that affects the rural population in Latin America. T helper cells type 17 (Th17) are described as an arm of the immune system that enhances host protection against several infections, including mycosis. Considering the better understanding of the mechanisms involved in resistance to *P. brasiliensis* infection is necessary, our aim was to evaluate the Th17 and related cytokines participation during the PCM.
Materials and methods: We worked with male C57BL/6 (WT) and IL-6, IL-17 receptor (R) and IL-23 knockout (KO) mice that were intravenously inoculated with 1×10^6 yeast forms of Pb18, a highly virulent *P. brasiliensis* strain. We measured cytokines mRNA and production from lung homogenate, besides, followed the fungal growth by recovering of colony forming units (CFU) and verified the inflammatory process and granulomas formation during de Pb experimental infection by cytometry and histopathology analysis.
Results: Our results show that Pb-infection induces increased IL-6, IL-17 and IL-23 mRNA lung expression and production compared with uninfected mice. Evaluating the susceptibility to the infection, the IL-6^{KO} mice showed the highest mortality rate. At 15 and 30 dpi, the CFU recoveries from the lung, liver and spleen of all KO mice were increased in relation to WT group. Histopathological analysis showed that IL-6 and IL-17 deficiency assisted absence of compact granulomas due to impaired formation of reticulin fibers accompanied of disorganized CD4$^+$ T cell infiltration at lung tissue, favoring an increased fungal load. The absence of IL-6, IL-17R or IL-23 induced lower production of IFN-g and IL-10 compared with WT lung. Additionally, the frequency of CD4$^+$IL-17$^+$ T cells also this cytokine production were decreased in the absence of IL-6 or IL-23.
Conclusions: Taken together, these results demonstrate that IL-6, IL-17 and IL-23 contribute to control of experimental Pb-infection through an efficient granulomatous organization.

P0966

Role of microglia and macrophages on retinal pigment epithelial cells degeneration and the factors influencing these in age related macular degeneration

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Purpose/Objective: Age related macular degeneration (AMD) is one of the common causes of blindness in elderly population. Retinal pigment epithelial (RPE) degeneration is one of the early events in AMD and these RPE cells mainly die through apoptosis. Maintaining a normal and functional RPE layer is vital for healthy vision. Therefore understanding the factors that induce RPE cell death in AMD would help identify a therapeutic approach. Inflammation is one of the key factors that influence the development of AMD. Infiltrating macrophages and resident microglia are associated with AMD and may influence RPE degeneration. Therefore we sought to identify the effects of cytokines, microglia and macrophages on RPE degeneration.
Materials and methods: An *in vitro* system to model *in vivo* conditions was set up by co-culturing murine macrophages derived from bone marrow cells or microglia from central nerves system on a confluent murine RPE cell layer under different conditions for 48 h at a ratio of 1:2 respectively. These conditions include RPE cells and macrophages or microglia pre-treated for 24 and 18 h respectively before the co-culture in the presence of pro inflammatory mediators, oxLDL, IL-1β, TNF-α, IL-6, and IFN-γ that are present in the microenvironment in which AMD develops. After co-culture the apoptotic cells were detected using annexin V staining by flow cytometry.
Results: Co-culture of un-treated RPE cells and un-treated BMDM or microglia does not increase apoptosis in RPE cells. However co-culture of un-treated RPE cells with pre-treated macrophages increased apoptosis in RPE cells but pre-treated brain microglia failed to increase apoptosis in RPE cells. Co-culture of pre-treated microglia and macrophages were able to increase apoptosis in treated RPE cells. These macrophages/microglia increase apoptosis in RPE cells through cell contact. In addition to macrophages and microglia, combination of cytokines and oxLDL also increases apoptosis in RPE cells.
Conclusions: The above findings provide a greater understanding of the pathogenesis of AMD, specifically the role played by microglia/macrophages in RPE degeneration and how oxidative stress and cytokines act together to bring about RPE degeneration. Better understanding of the pathogenesis of AMD will provide better therapeutic strategies.

P0967

S100B in the ocular inflammatory response; cytokine and chemokine modulation

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Purpose/Objective: S100B is a Ca^{2+} binding protein which can potentially act as a pro-inflammatory mediator via the receptor for advanced glycation end products (RAGE); however its involvement in inflammation is still unclear. In the human condition, Endogenous Posterior Uveitis, retinal inflammation with autoimmune aetiology is associated with loss of sight. Both S100B and RAGE are reported to be present in the retina and the purpose of the study was to determine whether S100B is involved in retinal inflammation.
Materials and methods: Using the mouse model, experimental autoimmune uveitis, Topical Endoscope Fundal Imaging allowed disease progression to be followed post-immunization (pi) with uveitogenic peptide, in S100B-deficient and wild type mice. Eyes were snapping frozen for histological grading and immunohistochemistry. Real-time PCR array analysis was used to confirm changes in cytokine and chemokine expression in diseased matched retinas from S100B-deficient and wild type mice. This method was also used to investigate the response of a murine macrophage cell line RAW 264 to treatment with S100B, with cytokine production confirmed by ELISA.
Results: We have shown using clinical grading, that disease is significantly reduced in S100B deficient mice compared to wild type, at days 15 and 21. At day 24 pi this disease reduction was also shown by histological grading of the infiltrate, although the composition of the infiltrate remained unchanged. In S100B knockout mice compared to wild-type, at day 24 pi there was an overall reduction in expression of proinflammatory cytokines and chemokines in diseased matched retinas. We confirmedthat the macrophage cell line express RAGE and that this expression increases upon activation with PMA. Treatment of the macrophages with S100B resulted in an increase in proinflammatory cytokines and chemokines, in particular IL-1β and CCL22. ELISA analysis confirmed a significant increase in CCL22 with 2 and 5 μM S100B compared to un-treated cells.
Conclusions: These results suggest that S100B may be involved in augmenting the inflammatory response in uveitis and this may be via a direct effect on macrophages.

P0968

The conformational significance of hinge region glycosylation on the solution structure of monomeric IgA1 in IgA nephropathy

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Purpose/Objective: Intrinsically characterised by the deposition of IgA1 in the renal mesangium, IgA nephropathy (IgAN) remains the most common form of glomerular inflammation worldwide. Within 20 years of diagnosis, up to 30% of patients progress to end stage renal disease. It is widely accepted that IgAN is caused by an inherent abnormality in the IgA1 molecule. Aberrant IgA1 hinge O-glycosylation, presenting as undergalactosylation, is observed in patients and is strongly implicated in the pathogenesis of IgAN.

Analytical ultracentrifugation (AUC) represents a powerful technique in the study of macromolecular size and shape in biologically relevant conditions. This study focuses upon the complex relationship between structurally induced changes in the IgA1 molecule through hinge region glycosylation, and its potential effects in the causation and prognosis of IgAN.
Materials and methods: Serum IgA1was isolated using jacalin affinity chromatography from a healthy control and 2 progressive IgAN patients. Monomeric IgA1 was separated by FPLC. The relative hinge region O-galactosylation was determined using a helix aspersa (HA) lectin binding assay including a standard curve containing serum samples with decreasing lectin binding. High lectin binding relates to low galactosylation.

AUC sedimentation velocity experiments were performed at 20°C with rotor speeds of 20 000 and 30 000 rpm for 16 h in PBS. Data was analysed using size-distribution plots c(s) using SEDFIT to determine monodispersity and establish sedimentation coefficients (S).
Results: The HA lectin binding values of the healthy subject and the patients 1 and 2, expressed as arbitrary units from a standard curve, were 22, 32 and 31, the sedimentation coefficients were 6.85 S, 6.7 S and 6.55 S respectively.
Conclusions: Sedimentation coefficient, a measure of molecular elongation increases when a molecule becomes more compact. The decrease in s value with increased HA binding and therefore decreased galactosylation, suggests a change in shape associated with the undergalactosylation of serum IgA1 in IgAN patients.

These results show that the conformational state of monomeric IgA may be directly altered via hinge glycosylation, potentially providing an implication to the increased deposition of IgA in IgAN.

P0969

The effect of interleukin-15 on inflammatory bone metabolism

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Purpose/Objective: Interleukin-15 (IL-15), mainly induced by macrophage or epithelium cell, is important for early response to compensate the time difference between innate immunity and adaptive immunity and deeply involved the initial immune response at local inflammatory site. However, it is still unclear about the effect on inflammatory bone metablism like periodontitis and peri-implantitis. Thus, we examine the effect of IL-15 on osteoblast and osteoclast differentiation.
Materials and methods: Bone marrow cells (BMCs) were collected from femurs of 4-week-old ddy mice. Osteoblasts from 2-day-old ddy mice were isolated from the calvaria. Osteoclast formation using primary cells was determined in co-cultures of osteoblasts (1.6×10^4 cells/well) and BMCs (4×10^6 cells/well). They were co-cultured for 7 days in a 24-well plate in the presence or absence of 10^{-6} M prostaglandin E_2 (PGE_2) and/or IL-15 (10, 100 ng/ml). To detect osteoclasts, the cells were stained for Tartrate-Resistant Acid Phosphatase (TRAP). TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. Osteoclast formation using cell line was determined in RAW 264 cells (6×10^3 cells/well). They were cultured for 5 days in a 48-well plate in the presence or absence of receptor activator of nuclear factor-κB ligand (RANKL) at 10, 25, 50 ng/ml. Osteoclast detection was carried out as described above.
Results: The number of osteoclasts in co-culture was decreased by IL-15 in a dose-dependent manner. The osteoblasts in co-culture seemed to disappear and be apoptotic cell death. This phenomenon was attenuated when T-cells and NK-cells were removed from BMCs. The number of osteoclasts in RAW 264 cells was increased by IL-15 in a dose-dependent manner.
Conclusions: IL-15 might promote inflammatory bone destruction by both promoting osteoclast differentiation and osteoblast apoptotic cell death. It is possible for IL-15 to be immunological therapeutic target for local inflammatory bone resorption like periodontitis and peri-implantitis.

P0970

The genes of AP-1 transcription complex as the potential factors which have an influencing on atherosclerosis development

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Purpose/Objective: Searching and research of the expression of the genes, which are connected with the risks of atherosclerosis development.
Materials and methods: It was used material of 34 autopsies taken from patients who were suffered from the ischemic attacks of the brain and acute heart failure. Mean age of the patients was 77.6 years old. It was used PubMed and UniGene bases during preparing the list of the genes. According to this list maps gene interactions were prepared. We looked throw the genes whose expression was changed more than 2 times. According to the maps key gene complexes are transcription factors AP-1 and NF-kB. NF-kB factor is not specific for the atherosclerosis process; therefore we have excluded it from research. Transcription factor AP-1 (Activating protein 1) is including proteins of Fos-, Jun- and ATF- families. Expression level of the components of AP-1 transcription factor at the blood vessel intimae was determined by Real-time PCR. Expression of the JunB, c-Jun, JunD, cFos genes was studied. Expression of the genes was normalized on the housekeeper gene GAPDH.
Results: cFos expression at the affected intimae of the blood vessel in comparison with the not affected intimae was increased in more than two times. JunB expression at the affected intimae was increased too. JunD expression in two patients was increased less than 2 times. In the case of 3 patients gene expression was increased more than 10. c-Jun expression decreased in 1 case. In the case of 2 patients' it was increased in more than 10 times. Thus, expression of the cFos, JunB, JunD, c-Jun genes in the in the most cases was increased.
Conclusions: Our data relate to those of other authors that the expression of the certain genes of the AP-1 transcription factor may vary in different pathological processes. Increasing of the level of cFos gene expression in 3 or more times is observed in patients, who were developed complications of atherosclerotic damage of the arteries which are supplying the tissue of the brain.

Our data suggest that the genes of AP-1 transcription factor are playing key role at the atherosclerosis processes.

P0971

The right dose determines a poison – influence of benzo(a)pyrene on the infection with *Salmonella enterica*

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Purpose/Objective: Polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene (BaP) are environmental contaminants exerting deleterious effects towards the immune system, ranging from immunosuppression to fatal inflammatory disease. Since exposure to BaP is ubiquitary, risk assessment was conducted to determine upper limits for tolerable daily intake doses (subtoxic concentrations). However, most studies demonstrating hazardous effects of BaP used concentrations in the toxic range. Therefore, the aim of this study was to investigate the influence of subtoxic BaP-concentrations on the outcome of an infection with *Salmonella enterica* (*S. e*) in a murine model.
Materials and methods: Mice were infected with *Salmonella enterica* and simultaneously exposed to subtoxic doses of BaP..
Results: Importantly, exposure to BaP in subtoxic concentrations markedly increased survival of mice after infection with *S. e.* However, BaP-treated animals developed a long-term infection with higher bacterial burden in spleen and liver when compared to vehicle controls. The number of B lymphocytes was significantly increased at day 90 post infection in BaP-exposed mice, which was accompanied by higher serum titers of *S. e.*-specific IgG1 and IgG2c antibodies. Furthermore, IL-17 and IL-22 mRNA levels in splenocytes were significantly up-regulated after long-term infection in BaP-treated mice.
Conclusions: Although BaP-treatment in subtoxic concentrations increased survival of mice during *S. e.* infection, it also promoted the survival of bacteria with a consequent increase in inflammatory responses. Our data suggest that constant BaP-exposure in subtoxic concentrations during an infection favours a bacteria-driven chronic inflammation.

P0973

Unravelling the causes of inflammatory steroid resistance via a systems biology approach

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Purpose/Objective: Steroid resistance is a generic problem which occurs in a number of autoimmune diseases. Treatment failure occurs in up to 30% of patients treated with steroids for inflammatory diseases. While it has been shown that there are significant homeostatic differences in cytokine and growth factor production profiles of interacting cell populations between steroid resistant and steroid sensitive cases, the mechanism of the steroid resistance is not understood. Recent evidence has implicated a steroid resistant CD4+ T cell subpopulation in the perpetration of steroid resistant disease.

Materials and methods: In order to investigate the inflammatory causes of steroid resistance we combined available experimental information into an extended systems model of our earlier work on interdependent cellular interactions. This project applies a systems based approach to the problem of steroid resistance using Steroid Resistant Nephrotic Syndrome (SRNS) as an example and is applicable to other steroid resistant cases.

Results: Using our unique cultured human glomerular cells and glomerular cell models we have measured cytokine and growth factor production in reponse to steroids using ELISA and mass spectrometry. These results allowed us to develop a model of the response of the kidney glomerulus to steroids. The model allowed the generation of a new hypothesis by predicting the appearance of pathological conditions as a result of altered cytokine and growth factor production rates and/or degradation rates in the glomerulus.

Conclusions: Our model predicts that glucocorticoids can modulate the cytokine concentrations and as a result of feedback loop interactions between cytokines and chemokines in a number of case may lead to the shift from pathologic conditions to healthy homeostasis. At the same time, we show that there can be cases when steroids can aggravate the disease and even introduce new disease etiologies.

Poster session: Hepatitis & Liver Immunology

P0974

Beneficial effects of Ocimum gratissimum aqueous extract on rats with CCl4-induced acute liver injury

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Purpose/Objective: Ocimum gratissimum (OG) is known as a food spice and traditional herb, which has been recommended for the treatment of various diseases. Herein we investigated the effects of OG leaf aqueous extract (OGAE) on reducing hepatic injuries in rats after CCl4 challenging.
Materials and methods: To investigate the hepatoprotective effect of OG aqueous extract (OGAE), male Wistar rats challenged by carbon tetrachloride (CCl4) were used as the animal model of chronic hepatic injury. Catalase assay (CAT) and DPPH assay was assessed in CCl4-administrated rats. Expression and phosphorylation of protein was determined and quantitated by immunoblotting using specific antibodies and densitometric analysis.
Results: Significantly increased serum catalase and DPPH levels were detected in CCl4-administrated rats that were treated with OGAE or sylimarin as compared to those rats that were treated with saline or CCl4. In contrast, significantly decreased stress proteins including HSP70 and iNOS were observed in livers of CCl4-administrated rats that were treated with OGAE or sylimarin as compared to those rats that were treated with saline or CCl4. Moreover, significant decreases of MMP-9/MMP-2 ratio, uPA, phosphorylated ERK (p-ERK) and NF-κB (p-P65) were detected in livers of CCl4-administrated rats that were treated with OGAE or sylimarin as compared to those rats that were treated with saline or CCl4.
Conclusions: These findings imply that OGAE can efficiently inhibit CCl4-induced liver injuries in rats and may therefore be a potential food or herb for preventing liver injuries.

P0975

CCR5-32 and UGT1A1 28− mutations in HCV patients

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Purpose/Objective: The role of CCR5-Δ32 and UGT1A1 28* mutations in HCV patients is not entirely clear, but it is known that CCR5-Δ32 contributes to immunology and UGTA1A 28* mutations cause Gilbert's syndrome which affects the liver. Presumably, a patient homozygous for both of these mutations is more likely to be at risk group for HCV infection. The objective to this study was to determine whether the occurrence of mutations CCR5-Δ32 and UGT1A1 28* is higher in HCV patients than in normal healthy group.
Materials and methods: To achieve our goal, we performed PCR for the genes in question and visualized the results in 6% polyacrylamide gel electrophoresis and by sequencing the PCR products. In this study we analyzed 262 individuals infected with hepatitis C and 187 healthy donors from general population, in total*243 male and 206 female individuals. For statistical analysis we used PLINK software, for biomedical association we used linear regression using additive model.
Results: Allelic frequency for CCR5-Δ32 mutation was 0.239 in HCV patient group and 0.176 in control group ($P = 0.0399$); for $(TA)^7$ allele it was 0.546 in HCV patient group and 0.355 in general population ($P = 0.00000005$.

For 79 patients there was more detailed information about their infection − viral RNA levels before and after therapy, viral genotype, iron, ferritin, ALT before and after therapy, viral clearance right after and 3 months after therapy. We found no significant association between patient's genotypes and RNA levels, ALT and iron levels ($P > 0.05$). Almost significant association was found between CCR5-Δ32 mutation and ferritin level by additive model (BETA -120.4, $P = 0.077$). Moreover, there was no association with viral clearance right after therapy, but 3 months after therapy for positive RNA CCR5-Δ32 allele frequency was 0.297, for negative − 0.0714 ($P = 0.0363$).
Conclusions: Patients with mutations in CCR5 and UGT1A1 are more likely to be infected with HCV, as their immune response is altered and their liver is weakened. It seems that UGT1A1 28* can affect liver function also in heterozygous state, but that way it does not cause Gilbert's syndrome. Presumably, the CCR5-Δ32 mutation correlates with unresponsiveness to therapy.

P0976

$CD39^+$ CD4+ T cells display a pro-inflammatory signature in patients with autoimmune hepatitis

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Purpose/Objective: Autoimmune hepatitis (AIH) is associated with numerical and functional regulatory T cell (Treg) defects. A recently described Treg subset expresses the ectonucleotidase CD39, which renders Tregs capable of suppression by initiating an ATP/ADP hydrolysis cascade culminating in the production of immunomodulatory adenosine. $CD39^+$Tregs exert preferential control over Th17 cells, an effector subset involved in AIH liver damage. CD39 is also present on a proportion of CD4 memory lymphocytes with effector function. Regulatory and pro-inflammatory features of $CD39^+$ $CD4^+$ cells in AIH are untested. We therefore sought to provide a phenotypic signature of $CD39^+$ $CD4^+$ cells in AIH.
Materials and methods: Of the 14 AIH patients and 6 healthy subjects (HS) were studied. Circulating $CD39^+$ $CD4^+$ cells were phenotyped by flow cytometry using monoclonal antibodies to CD4, CD39, CD25, FOXP3, Granzyme B, CD62L, CTLA-4 and RORC. The frequency of TGFβ and IL17A-producing cells was assessed by intracellular cytokine staining after incubation with Leukocyte Activation Cocktail (LAC).
Results: Compared to $CD39^-$ $CD4^+$ cells, a higher proportion of $CD39^+$ $CD4^+$ cells expressed Treg and Th17 markers in AIH and health. Compared to HS, $CD39^+$ $CD4^+$ cells in AIH patients contained fewer cells positive for the Treg-associated markers CD25, FOXP3, and Granzyme B, while CD62L or CTLA-4 positive cell frequencies were similar. After LAC stimulation, the frequency of TGFβ-producing cells among $CD39^+$ $CD4^+$ cells was lower in AIH patients compared to HS. In contrast to Treg markers, $CD39^+$ $CD4^+$ cells from AIH patients contained a higher proportion of cells positive for the Th17 transcription factor RORC than those from HS. There was no difference in the proportion of IL17A-producing cells within $CD39^+$ $CD4^+$ cells between AIH and HS.
Conclusions: The $CD4^+$ population expressing the ectoenzyme CD39 contains a high proportion of cells expressing Treg and Th17 markers. In AIH the lower frequency of cells positive for conventional Treg markers and the higher number of cells positive for RORC indicates that the $CD39^{++}$ $CD4^+$ population is skewed towards a pro-inflammatory phenotype. Whether the effector potential of Th17 committed lymphocytes is mitigated by the presence of CD39 awaits investigation.

P0977

CD8ßlow – a novel differentiation marker and a prominent population in chronic hepatitis B infection

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Purpose/Objective: Failure of antigen-specific CD8 T cells is a recognised feature of chronic hepatitis B (HBV) infection, however the bulk CD8 T cell population is also characterised by low expression of CD28 and poor IL-2 production and proliferation capacity (Hoare D *et al.* 2008) in keeping the phenotype of late differentiated CD8 T cells. We have previously observed a prominent CD8 T cell population in chronic HBV to be CD8α+β^{low} (Kang W *et al.* 2009) – this is most obvious in the cells which either lack (CD161-) or have low expression of CD161 (CD161+) (a molecule associated with liver homing). In this study we aimed to explore the relationship between these two observations in chronic HBV, chronic Hepatitis C (HCV) and healthy controls (HC).

Materials and methods: Peripheral blood mononucleocytes were obtained from patients with chronic HBV (n = 37), chronic genotype 1HCV (n = 24) and healthy controls (n = 15). All patients were treatment naïve. FACS analysis was performed on both cell surface antibody staining using a panel of activation/exhaustion/differentiation markers (CD25, CD38, CD69, HLA-DR, PD-1, CD8α, CCR7, CD62L, CD45RA, CD45RO, CD28, CD27, CD57) and intracellular cytokine staining following PMA/ionomycin stimulation.

Results: Prominent populations of CD161$^-$ and CD161+ CD8α+β^{low} T cells can be identified in healthy individuals as well as those with chronic viral hepatitis, however a significantly greater population of CD161-/CD161$^+$ CD8α^+ CD8β^{low} T cells is seen overall in chronic HBV compared to healthy controls (mean 39.5% HBV, mean 24.74% HC, P = 0.05). The CD8α+β^{low} T cells have the CD28$^-$ CD27$^-$ CD57$^+$ CD62L$^-$ CCR7$^-$ CD45RA$^-$ phenotype of late differentiation in both healthy controls and patients with chronic HBV and HCV and express high levels of the late activation marker HLA-DR. CD161- CD8α+β^{low} CD8 T cells produce significantly more IFN-γ and TNF-α on stimulation with PMA/ionomycin than their CD161$^-$ CD8α+β^{high} counterparts and express greater levels of Ki67 and perforin.

Conclusions: CD8β^{low} status represents a novel marker for late differentiated CD8+ T cells and may alter the ability of CD8+ T cells within this population to respond to CD8$\alpha\beta$ dependent epitopes. The prominence of CD8α+β^{low} T cells in chronic HBV infection is likely to profoundly influence the immune environment, with important implications for the development of immunotherapy and treatment.

P0978

Cell-targeting gold nanorods critically affect the outcome of liver inflammation *in vivo* by modulating macrophage polarization

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Purpose/Objective: Hepatic macrophages critically promote liver inflammation and fibrogenesis. Thus, hepatic macrophage-specific immunomodulatory nanoparticles represent a promising therapeutic option in liver diseases. We reported recently that human primary macrophages are strongly polarized by nanoparticle surface chemistry *in vitro*. Here, we investigated the toxicity, distribution, and therapeutic effects of gold nanorods (AuNR) in acute and chronic liver injury *in vivo*, aiming to develop novel therapeutic strategies for the treatment of liver disease.

Materials and methods: We studied the concentration-dependent organ distribution of AuNR coated with either CTAB, PEG, or with the tripeptides GLF, or RGD *in vivo* in C57BL/6J mice using inductively coupled plasma-mass spectrometry, electron microscopy, and *in vivo* imaging (micro-CT). The therapeutic effects of the AuNR were studied in Concanavalin A (ConA)-mediated acute hepatitis as well as in carbon tetrachloride (CCl_4)-induced fibrosis, using biochemistry (ALT, AST, hydroxyproline), histology, immunohistochemistry, and flow cytometry. Macrophages were isolated from liver and analyzed for polarization by qPCR-based assays.

Results: We found that the AuNR predominantly accumulate in liver *in vivo*, specifically in hepatic macrophages. At high concentrations (1200 μg/kg), CTAB-coated AuNR slightly increased infiltrating inflammatory CD11b$^+$F4/80$^+$Gr1^{high} macrophages in the liver without evidence of hepatotoxicity *in vivo*. Interestingly, upon ConA-induced hepatitis, RGD-peptide-modified AuNR significantly deteriorated liver damage, decreased the frequency of resident CD11b$^-$ F4/80$^+$Gr1^{low} Kupffer cells accompanied by strong changes in the expression of surface markers and function-related genes expressed by classically or alternatively activated macrophages, compared to untreated controls. Similar observations were found in chronic liver injury, with a trend towards increased liver fibrosis development by RGD-coupled AuNR.

Conclusions: AuNR efficiently target hepatic macrophages, and modifications of nanoparticle surface chemistry affect the phenotype and functionality of both liver infiltrating and resident macrophages, thereby impacting the hepatic response towards acute or chronic injury.

P0979

Characterization of two human monoclonal anti-E2/HCV antibodies for potential application in prevention and treatment of HCV infection

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Purpose/Objective: HCV is a major cause of chronic liver disease worldwide and the current antiviral therapies results successful in approximately half of treated patients. Liver disease caused by HCV infection is a common indication forliver transplantation but graft re-infection is universal and often results in graft loss. Immunotherapies employing neutralizing antibodies, able to inhibit HCV infection, directed against the HCV viral glycoproteins E1 and E2, involved in cell viral entry, have the potential to control or prevent graft reinfection. Moreover, anti-HCV neutralizing antibody can find a potential application for the treatment of chronically infected patient.

Here we characterized two potent neutralizing anti-E2/HCV human monoclonal antibodies (mAbs).

Materials and methods: We previously characterized two anti-HCV E2 human mAbs as Fab fragments, e20 and e137. To obtain molecules more suitable for therapeutic purposes, we generated whole IgG e20 and e137. Both mAbs were tested in ELISA and immunofluorescence assay (IF) to study the affinity and the reactivity on different genotypes. To determine the capacity of these antibodies to interfere with E2-CD81 binding, we performed an inhibition of binding (IOB) of CD81 to recombinant E2 glycoprotein by ELISA. For studying biological activity of e20 and e137, neutralization assay against HCVpseudo-

particles of five genotypes and cell culture infectious HCV system based on genotype 2a was performed.
Results: The antibodies showed high affinityfor E2 recombinant glycoprotein (genotype 1a) and were able to recognize HEK293T cells expressing HCV E1-E2 from the principal HCV genotypes. Both IgGs showed dose dependent IOB activity and at the maximum tested concentration the inhibition was about 97%. Neutralization assay showed that these antibodies are strong neutralizers of the tested genotypes with IC50 ranging from 0.02 to 2 μg/ml.
Conclusions: e20 and e137 are potent cross-reactive and cross-neutralizing mAbs able to inhibit E2-CD81 binding. These data suggests that both antibodies are directed against conserved and critical residues for viral infectivity on E2 glycoproteins. Based on their features, both IgGs may assist in the development of an effective passive immunotherapy for the prevention of viral reinfection of the liver graft and treatment of chronically infected patients.

P0980
Chemokine presentation by liver sinusoidal endothelial cells as therapeutic target in murine T cell-mediated hepatitis

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Purpose/Objective: Leukocyte adhesion and transmigration is one of the central paradigms of inflammation. Within the liver sinusoids, chemokines initiate the first crucial step of lymphocyte migration into the parenchyma. Especially liver sinusoidal endothelial cells (LSEC) present chemokines to T cells increasing their biological activity. We here investigated mechanisms of chemokine transport by LSEC and whether inhibition of endothelial chemokine presentation influences autoimmune hepatitis.
Materials and methods: In the murine model of Concanavalin (Con) A-induced T-cell mediated hepatitis chemokine expression was determined. Chemokine uptake, transcytosis and presentation by LSEC were visualized by confocal microscopy and functionally investigated in transmigration assays. Effects of reduced endothelial chemokine presentation on the development of hepatic inflammation were monitored by alanine transaminase and histology.
Results: During Con A-induced hepatitis, liver mRNA expression of the pro-inflammatory CXC chemokine ligand (CXCL)9 and CXCL10 was significantly increased. Furthermore, CXCL9 was particularly shown within LSEC. LSEC internalized basolateral CXCL9, CXCL10 and CXCL12 via clathrin-coated vesicles and provided these chemokines immobilized on the glycosaminoglycans heparan sulphate and chondroitin sulphate to $CD4^+$ T cells, thereby increasing transmigration *in vitro*. Blockage of the clathrin-dependent cellular transport pathway significantly reduced endothelial chemokine internalization and consequently chemokine-dependent $CD4^+$ T-cell transmigration across LSEC. Administration of a clathrin inhibitor *in vivo* suppressed Con A-induced autoimmune hepatitis and decreased accumulation of activated $CD4^+$ T cells expressing the CXC chemokine receptor 3.
Conclusions: Intervention in endothelial chemokine provision *in vivo* affects chemokine-dependent migration of pro-inflammatory $CD4^+$ T cells into the liver, thereby counteracting development of autoimmune hepatitis. Thus, chemokine presentation by LSEC during liver inflammation might be a promising therapeutic target in hepatitis.

P0981
Corticosteroids affect hepatitis C infection by modulating plasmacytoid dendritic cells but not interferon-a signaling

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Purpose/Objective: Chronic hepatitis C virus (HCV) infection is one of the leading indications for liver transplantation, but outcomes are often compromised by re-infection of the graft. Several studies have indicated that the use of corticosteroid-based immunosuppression is a risk factor for severe HCV recurrence. The mechanism for the steroid-mediated effect on HCV is not fully elucidated, but recent studies using *in vitro* HCV models found no direct effect on viral replication. The success rate of interferon-a (IFN-α) based antiviral therapy is significantly lower post-transplantation than in the non-transplant HCV population; however the impact of steroids on the antiviral activity of IFN-α is unknown. Therefore, the aim of this study is to investigate the effect of steroids on the antiviral activity of IFN-α and the impact on the primary IFN-α-producing cells, the plasmacytoid dendritic cells (PDCs).
Materials and methods: As a model for HCV replication we used the Huh7 hepatoma cell line, stably transfected with the non-structural coding sequence of HCV directly coupled to a luciferase reporter gene (Huh7-ET), and treated with IFN-α in the presence or absence of different doses of prednisolone or dexamethasone. A Huh7 cell line stably transfected with a luciferase gene under the control of an interferon response element (Huh7-ISRE-Luc) was used to investigate effects on IFN-α signal transduction. To investigate the effects of steroids on PDCs, Huh7-ET cells were co-cultured with human PDCs in the presence or absence of steroids.
Results: HCV replication was inhibited by 10 IU/ml IFN-α by more than 99% of control levels. Treatment with increasing doses of dexamethasone or prednisolone did not significantly affect HCV replication. When combining IFN-α with dexamethasone or prednisolone, no interference with the inhibition of HCV replication by IFN-α was observed. Moreover, dexamethasone and prednisolone had no effect on IFN-α signal transduction as measured in Huh7-ISRE-Luc cells. However, when Huh7-ET cells were co-cultured with PDCs, a significant reduction of HCV replication was observed, which was almost completely reversed by treatment with steroids.
Conclusions: We found no evidence that corticosteroids interfere with signal transduction and antiviral action of IFN-α. However, steroids may affect HCV replication post-transplantation by reducing the antiviral capacity of PDCs.

P0982
Differential migration patterns of CD8 T cells primed in the liver and gut

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Purpose/Objective: Patients with inflammatory bowel disease (IBD) are frequently affected by autoimmune liver diseases, suggesting that these anatomical distinct diseases may share pathogenic features. Since IBD is T-cell mediated, it is possible that T cells activated in the gut migrate to the liver where they cause inflammation. In earlier experiments we have shown that gut- as well as liver-activated T cells

accumulate in the liver, but only gut-activated CD8 T cells are licensed to migrate to the small intestine. In contrast liver-activatedCD8 T cells retain the capacity to migrate through lymph nodes. Here we analysed the expression of activation and adhesion molecules of these differentially activated T cells, which may explain their migration behaviour.
Materials and methods: Antigen-specific naïve OT-I CD8 T cells were adoptively transferred into mice, in which ovalbumin is expressed in the liver (TF-OVA mice) or in the small intestine (iFABP-OVA mice). Effector T cells were isolated from liver and mesenteric lymph nodes after three days. Activation assays as well as microarray analysis and flow cytometry were used to examine distinct or shared imprinted patterns of these cells.
Results: Transcriptome analysis of naïve, liver- and gut-activated CD8 T cells identified 10 326 differentially regulated IDs. Hierarchical clustering clearly discriminated naïve, liver- and gut-activated T cells. Activation in the gut induced the typical gut-specific CCR9/$\alpha4\beta7$ T cells. In contrast liver-activated T cells displayed increased expression of $\alpha4\beta1$, $\alpha6\beta1$, Ly6C, PD-1 and CD62L, whereas activation markers as well as $\alpha L\beta2$ were upregulated in both cell types. The adhesion molecules ICAM-1 and VCAM-1 are constitutively expressed in the liver and were upregulated upon inflammation, whereas MAdCAM-1 expression could not be detected in the liver.
Conclusions: Liver-activated CD8 T cells display a unique phenotype compared to naïve and gut-activated T cells. High expression of CD62L and Ly6C may be responsible for their migration through lymph nodes. Only gut-activated T cells show gut-specific migration, but they also accumulate in the liver. This accumulation probably results from their adhesion to VCAM-1 and ICAM-1 in the liver. The migration of activated CD8 T cells is a one way route from the gut to the liver and may be responsible for the development of liver disease in patients with IBD.

P0983

Estudy of LDL influence in the interaction between HCV E2 protein and the surface receptors of ECV304 cell

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Purpose/Objective: The HCV presents a world-wide prevalence of 3%, being the Hepatitis C one of the most important public health problems. The virus is enveloped and presents 10 different structural and non-structural proteins, amongst them the envelope 2 glycoprotein (E2). The E2 protein presents a strong association with the LDL receptor, suggesting that the HCV may use it to invade cells. An association between HCV and LDL from human serum was demonstrated in preliminary studies which report that lipoproteins may increase HCV infectivity. The aim was to produce a recombinant protein similar to HCV E2 protein, codified by partial E2 protein gene (without TM domain) and not glycosylated, to analyze its binding to the surface receptors of ECV304 cells, in the presence and absence of human LDL, in order to verify the influence of LDL in this binding process.
Materials and methods: The coding gene of the E2-like protein fused to the GST was cloned in the pET-42a vector and transformed into *E. coli* bacteria strain Rosetta, which was induced to protein expression with IPTG, at 37°C, 300 rpm for 3 h. The proteins were purified by glutathione column. ECV304 cells were incubated at 4°C for 90 min with 20 μg of E2 protein only and with 20 μg of E2 protein added of 40 μg of human LDL. The cells were washed with PBS pH7.2 and incubated at 4°C for 30 min with anti-his antibodies conjugated with APC. The cells were washed with PBS pH7.2 and submitted to analysis in *FACS Canto BD* cytometer, *software Diva FACS*..
Results: The flow cytometry results showed that the E2 recombinant protein bound to 20% of the cells and the proteins with human LDL bound to 35% of the tested cells.
Conclusions: The flow cytometry revealed that the addiction of human LDL to E2 recombinant proteins provided an increase of 75% in the binding to surface receptors of ECV304 cells, phenomenon that may increases the HCV infectivity.
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P0984

Expression and function of Toll-like receptors in human hepatic stellate cells

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Purpose/Objective: Human hepatic stellate cells (HHSC) play pivotal role in chronic liver diseases. They are involved in the induction and progression of hepatic fibrosis leading to liver cirrhosis. Toll-like receptors (TLRs) are known to be expressed on/in HHSC and some of them, such as TLR4 have been claimed to promote liver fibrosis. Presence and function of other TLRs in HHSC remains totally inknown. The aim of the current study was to to get some information about the expression and function of these structures in the cells in question.
Materials and methods: Established cell line of HHSC, LX2 was used. Cell cytospins were searched by immunocytochemistry (ICH) and cell suspensions by flow cytometry (FC) for TLR1-10 expression, as a per cent of positive cells and mean fluorescent intensity (MFI). RNA was isolated from cells and tested for mRNA specific for respective TLRs. Cells were subjected to short time culture with appropriate TLR ligands and afterwards assessed by FC for TLR expression. Culture supernatants were searched for a number of released cytokines by Flexnet (BD) cytometric assay.
Results: LX2 cells were shown to express all TLR1-10, both, tested by ICH and FC, but the per cent of positive cells varied. TLR mRNA was evidenced also for all TLRs tested. Culture with ligands resulted usually in lower MFI expression than in medium alone. SSpolyU, TLR8 ligand was the only one, that provided slightly higher MFI value of LX2 cells as compared to medium alone. The only cytokine, or rather chemokine, produced by LX2 cells in reasonable amounts (up to 800 pg/ml) was stromal cell-derived factor-1 (SDF-1a also known as CXCL12). Its production however appeared to be constitutive feature of LX2 cells, because it was also secreted by cells cultured without any TLR ligands. Another cytokine, being also a chemokine, that was secreted by cells in tiny but measurable range (53 pg/ml) was IL-8. It was the case, when cells were cultured in medium supplemented with FLA-BS, ligand of TLR5. Besides, cells cultured in medium (without any ligand) secreted TGF β_1, in amounts of 96 pg/ml.
Conclusions: Expression of TLRs (1−10) appears to be common phenomenon on/in LX2 cells. Their activation by TLR ligands has relatively modest effect on TLR expression and function. Presumably, in the case of HSC *in vivo*, these cells are boosted by other, hitherto unknown receptors or agents to manifest their profibrotic activity.

P0985

HCV Core protein induced immune disordre via oxidative stress and thiol redox alteration

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Purpose/Objective: The network linking HCV infection, inflammation, free radical production, and carcinogenesis applies very well to HCV-mediated chronic liver damage, just as it applies to any chronic inflammatory condition. Research into the role of structural and nonstructural proteins of HCV and the changes induced in cytokine expression oncogenes, antioncogenes, and intracellular kinases shows that HCV is by itself and not only through inflammation able to induce ROS, an effect specific to this virus. This free radical production, accompanied by oxidative genomic injury, constitutes the first step of a cascade of genomic and postgenomic events that play an important role in HCC. More information is necessary from recently introduced technologies for proteomics that will hopefully close the gap between hypothesis and understanding. In the present study, we provide further evidence in support of this postulate. The technique used in our search, combining proteomics and proteine redox modification by S-glutathionylation, can help identifying proteins specifically modified under HCV Core pathological conditions.
Materials and methods: In the present work, we have engineered lentiviral vectors expressing the HCV core to determine how the pattern of protein oxidation is affected by disrupting thiol reduction pathways in CD4 Jurkat cells expressing HCV core protein. As a step towards characterizing relative susceptibilities of cell proteins to oxidation and identifying what changes can altere signalling or metabolic regulation, we have taken a proteomic approach (mono and two-dimensional electrophoresis; and MALDI-TOF MS) to identifying thiol proteins that become oxidized in both CD4 Jurkat cells expressing HCV Core and JKT cells exposed to oxidative stress (H_2O_2); This model was previously reported to cause extensive S-thiolation of cellular proteins. we also monitored oxidized thiols *in situ* by a technique that involves labelling with the fluorescent probe and analyzed by flow cytometry in a CyanADP-MLE (DakoCytomation) using an UV enterprise laser set at 30 Mw.
Results: Core protein increased S-glutathionylation in CD4 Jurkat cells. These results suggest that expression of core protein and subsequent oxidation of the glutathione pool and oxidative cysteine modifications inducing glutathiolation may be an important cause of the Alteration of immune cell function and the progression from chronic hepatitis C to HCC.
Conclusions: This new insight into the mechanisms for HCV mediated immune evasion and lymphocyte homeostasis alteration may offer novel therapeutic targets for one of the most devastating human malignancies in the world today

P0987

Hepatocytes mediate Notch dependent immune regulation in response to inflammation in the regenerating mouse liver

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Purpose/Objective: A single injection of concanavalin A (ConA) to mice induces acute Th1-mediated hepatitis. Tolerance against ConA rechallenge develops within 8 days and is mediated by IL-10 predominantly produced by $CD4^+$ $CD25^+Foxp3^+$ regulatory T cells (Tregs) and Kupffer Cells. This study was intended to identify the role of hepatocytes (HC) in ConA-induced immunoregulation.
Materials and methods: HC or splenic DC were isolated from saline- or ConA-pretreated wt, IL-10-, IFNγ-, or interferon regulatory factor (IRF)-1-deficient mice. Subsequently, HC or DC were co-cultured with splenic $CD4^+$ T cells from wt, DEREG or IL-10 KO mice and stimulated with anti-CD3 for 60 h. Cytokine release was measured by ELISA. Frequencies of $CD4^+Foxp3^+$ Tregs and $Notch1^+$ T cells were quantified by FACS analysis.
Results: Naive $CD4^+$ wt T cells co-cultured with wt HC from ConA-tolerant mice showed significantly increased IL-10 levels indicating a tolerant phenotype. T cells were identified as major source of IL-10. In contrast, splenic DC from ConA-tolerant mice failed to induce IL-10 release in naïve wt T cells. $CD4^+$ wt T cells co-cultured with ConA-primed IFNγ- or IRF-1-deficient HC released significantly lower levels of IL-10 compared to co-cultivation with wt HC from ConA-tolerant mice. Moreover, the γ-secretase inhibitor DAPT, which blocked Notch activation, prevented IL-10 secretion. Interestingly, HC from ConA-tolerant mice increased the frequency of $CD4^+Notch1^+$ T cells as well as receptor density of Notch1 on $CD4^+$ T cells. Furthermore, Foxp3 expression was elevated in co-cultures containing T cells from DEREG mice and ConA-tolerant HC compared to non-primed HC. Moreover, HC from ConA-tolerant mice promoted the TGFβ-driven conversion of naïve wt T cells to $Foxp3^+$ Tregs which was again abrogated by DAPT.
Conclusions: We showed that HC from ConA-tolerant mice induce an IL-10-secreting regulatory phenotype in naïve T cells and convert these cells into $Foxp3^+$ Tregs in the presence of TGFβ. The conversion depends on an intact IFNγ-dependent Th1 response and on Notch signalling. The failure of splenic DC to induce IL-10 expression indicates that the generation of an IL-10 producing T cell subset is restricted to liver-resident non-professional APCs favouring the 'liver tolerance effect' as well as regeneration in response to inflammation.

P0989

Human cytomegalovirus infection of human hepatic sinusoidal endothelial cells promotes CD_4 T cell recruitment and post-transmigrational activation

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Purpose/Objective: Animal studies suggest that endothelial cells and not hepatocytes are the site of cytomegalovirus (CMV) latency and reactivation in the liver and the source of secondary viral spread. Furthermore, murine CMV infection of sinusoidal endothelium is able to break immunotolerance and induce a strong CD8 T cell effector response. The aim of this study was to investigate, whether CMV infection of human hepatic sinusoidal endothelial cells (HSEC) modulates the ability of the liver to recruit and activate CD4 lymphocytes.
Materials and methods: Recombinant endotheliotropic eGFP-labeled CMV was propagated in RPE-1 cells and purified by ultracentrifugation in tartrate/glycerol gradients. Primary HSEC were isolated from explanted livers, grown to confluence and infected with CMV over 2 h. Infection was confirmed by fluorescence microscopy and plaque assay on fibroblasts. Expression of adhesion molecules and costimulatory on infected HSEC were analyzed by flow cytometry and cell-based elisa. Resting CD4 T cells, regulatory T cells and CMV-specific CD4 clones were perfused over HSEC monolayers 24 h after infection under constant flow simulating physiological shear stress and adhesion and transmigration recorded using phase contrast microscopy. Static transmigration assays through HSEC into collagen were used to study phenotype and cytokine production of transmigrating lymphocytes using flow cytometry.

Results: Human sinusoidal endothelial cells were permissive to CMV infection. CMV infection of HSEC resulted in an increase of ICAM-1 and a decrease of ICAM-2, PD-L1 and CD40 and secretion of CXCL10 and CCL5. Under flow, transendothelial migration of CD4 T cells was increased through CMV-infected endothelium and predominantly mediated by ICAM-1. Transmigrated allogeneic CD4 effector memory T cells from CMV-seropositive and seronegative donors displayed a strongly increased expression of CD69 at 24 h and CD25 after 48 h after transendothelial migration through CMV-infected HSEC into collagen and demonstrated a Th1 phenotype (T-bet+, IFNg+, TNFa+) independently from HSEC MHC class II expression.
Conclusions: CMV infection of HSEC facilitates the up-regulation of cell-adhesion molecules and chemokines resulting in increased adhesion, transmigration and activation of CD4 T cells. This may explain how human CMV infection not only provokes significant hepatitis but also increases hepatic immune activation in graft rejection.

P0990

IL-33 exacerbates liver ischemia-reperfusion injury in mice

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Purpose/Objective: The aim of this study was to examine the role of IL-33 on liver I/R injury in mice.
Materials and methods: A partial lobar liver warm ischemia model was performed. The expression of IL-33 and ST2 were analysed in sham mice and liver I/R injury mice, using real-time polymerase chain reaction, western blotting, immunohistochemisry staining and confocal microscope approaches. The liver function, the level of cytokines and TLR4 and the infiltration of neutrophils were measured in sham, anti-IL-33 antibody, rIL-33 protein and saline group by ELISA, flow cytometry, immunohistochemisry staining, respectively.
Results: Our results show that both mRNA and protein levels of IL-33 were overproduced in I/R injury mice but not sham mice ($P < 0.05$). The major sources of IL-33 during liver I/R injury were injury hepatocytes and the vascular endothelial cells. Those mice pre-treated with anti-IL-33 antibody 1 h before ischemia showed decreased serum alanine aminotransferase levels, inhibited production of proinfiammatory cytokines such as IL-1β, IL-18, TNF-a, and IL-6 as well as decreased of infiammatory cell infiltration by down regulation of TLR4 on kupffer cell, leading to the prevention of liver I/R injury, when compared with controls ($P < 0.05$). Histology revealed that pre-treated with anti-IL-33 antibody significantly ameliorated hepatocellular damage ($P < 0.05$). At the same time, those mice pre-treated with rIL-33 protein revealed more serious liver injury than control mice ($P < 0.05$).
Conclusions: our study confirms that IL-33/ST2 signalling is involved in liver I/R and that inhibition of IL-33 can protect the liver from I/R injury by reducing IL-1β, IL-18, TNF-a, IL-6 release and infiammatory cell infiltration through down regulation of TLR4 expression on kupffer cell.

P0991

Immune cell CD39 and CD73 expression in autoimmune hepatitis and health

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Purpose/Objective: CD39 is an ectoenzyme that works in tandem with CD73 to degrade ATP and ADP into AMP and immunosuppressive adenosine. In mice, $CD4^+$ $CD25^{high}$ regulatory T cells (Tregs) express both CD39 and CD73. In humans $CD39^+$ Tregs have potent immunomodulatory capabilities, despite variable CD73 expression. Autoimmune hepatitis (AIH) is an inflammatory liver disorder characterised by reduced Treg frequency and function. CD39 and CD73 expression by different immune cell subsets has not been fully characterised. Our objective was to determine the frequency of circulating $CD39^+$ and $CD73^+$ immune cells in AIH and health.
Materials and methods: Twenty AIH patients and 10 healthy subjects were studied. The frequency of $CD19^+$ (B cells), $CD11c^+$ (dendritic cells), $CD8^+$, $CD56^+$ (natural killer cells), $CD4^+$ $CD25^-$ and Treg cells expressing CD39 and CD73 was measured by flow cytometry.
Results: $CD19^+$ and $CD11c^+$ populations contained the highest frequency of $CD39^+$ cells in both AIH patients and HS. CD39 was expressed to a lesser extent by Tregs, and the frequency of $CD39^+$ Tregs was lower in AIH than in HS. The frequency of $CD39^+$ cells within the $CD4^+$ $CD25^-$, $CD56^+$ and $CD8^+$ subsets was low in both groups, but $CD39^+$ $CD8^+$ lymphocytes were fewer in AIH than in HS.

The $CD19^+$ and the $CD8^+$ subsets contained the highest frequencies of $CD73^+$ cells in both groups, with $CD73^+$ $CD8^+$ lymphocytes being more numerous in AIH than in health. A lower proportion of Tregs from both groups were positive for CD73. The frequency of $CD73^+$ cells within $CD11c^+$, $CD56^+$ and $CD4^+$ $CD25^-$ populations was low in both AIH and health, but AIH patients displayed higher frequencies of $CD73^+$ $CD56^+$ and $CD73^+$ $CD4^+$ $CD25^-$ lymphocytes than HS.
Conclusions: Circulating immune cells display differential CD39 and CD73 expression patterns. In AIH, the low proportion of $CD39^+$ cells within Tregs and $CD8^+$ lymphocytes may account for impaired immune-regulation. Both in AIH and health, only a small proportion of Tregs are positive for CD73, suggesting that completion of the ectonucleotidase cascade is performed by $CD19^+$ and $CD8^+$ cells, the higher frequency of $CD73^+$ cells in AIH possibly reflecting an inflammatory state. Since $CD19^+$ cells express the highest frequency of both CD39 and CD73, their possible role as regulatory B cells needs to be investigated in future studies.

P0992

Immunohistochemical study of Toxocara-induced hepatic inflammation: characterizing the immune response players

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Purpose/Objective: The aim of this study was to characterize the key immune cells and the rate of apoptosis in hepatic inflammation during the course of experimental infection by Toxocara canis.
Materials and methods: Mice experimentally infected with Toxocara canis were divided into two groups: mice with primary infection by Toxocara, and those infected after sensitization by Toxocara excretory-secretory antigen. CD4+, CD8+, andBcl-2-expressing T lymphocytes were identified in the liver by immunohistochemistry at different durations post-infection.
Results: We observed recruitment in both CD4+ and CD8+ T lymphocytes with difference in count and localization within the liver. These cells were detected within and around Toxocara-induced granulomas as well as in isolated inflammatory foci in the portal tracts

or within the hepatic parenchyma. The antiapoptotic protein Bcl-2 showed no significant change at different periods post-infection. On the other hand, immunization of mice with Toxocara excretory-secretory antigen prior to experimental infection caused earlier and more pronounced recruitment of CD8+ T cells to the liver and enhanced expression of Bcl-2.

Conclusions: These results suggest a dynamic change in key immune cells according to duration of infection as well as the immune status of the host.

P0993

Induction and functionality of hepatic regulatory CD4+ T cells

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Purpose/Objective: Liver sinusoidal endothelial cells (LSEC) play an important role in shifting local immune responses to tolerance in major histocompatibility complex (MHC) I-restricted models of antigen presentation. Their impact in MHCII-mediated antigen presentation in the context of tolerance and immunity is still under investigation. Recently, in a bone marrow chimeric mouse model expressing MHCII exclusively on non-hematopoietic cells like LSEC, the induction of $CD4^+$ $CD25^{low}$ forkhead box protein (FoxP)3$^-$ regulatory T cells by LSEC has been described.

Materials and methods: In a model of T cell-mediated autoimmune hepatitis, we adoptively transferred OVA-specific $CD8^+$ and $CD4^+$ T cells alone or in combination with LSEC-primed $CD4^+$ T cells (T_{LSEC}) into TF-OVA mouse, expressing OVA exclusively in the liver. The suppressive capacity of T_{LSEC} was analyzed by CD8 T cell suppression assay *in vitro*. We further investigated the mechanisms involved in induction of T_{LSEC} phenotype. Transfer colitis model was used to gain insights into the ability of regulatory T_{LSEC} to suppress the development and progression of intestinal inflammation.

Results: T_{LSEC} suppress a T cell-mediated autoimmune hepatitis *in vivo*. *In vitro* T_{LSEC} have the capacity to suppress proliferation, activation and development of effector molecules of $CD8^+$ T cells. We investigate mechanisms involved in the induction and function of regulatory $CD4^+$ T cells by LSEC. Especially retinoic acid seems to play an important role during induction of T_{LSEC} phenotype. Surprisingly, T_{LSEC} do not only home into the liver but also migrate into the gut, enabled by expression $\alpha_4\beta_7$ integrin and CC chemokine receptor (CCR)9. Initial experiments showed an inhibitory effect of T_{LSEC} on intestinal inflammation by reduced lamina propria lymhocyte cell counts and a slightly reduced clinical colitis score.

Conclusions: The suppressive capacity and gut-homing properties of T_{LSEC} open for the intriguing possibility that recruitment of liver-primed $CD4^+$ T cells into the intestine might modulate the immunological balance of the gut.

P0994

Induction of human CD14+HLA-DR- Myeloid Derived Suppressor Cells by Hepatic Stellate Cells

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Purpose/Objective: Tumors have evolved different mechanisms to evade the host immune response and generate a suppressive network. In addition to regulatory T cells (Tregs) and tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) are of great importance and are becoming a focus of interest.

The only possibility to overcome this problem will be a careful phenotypical and functional analysis of all potential MDSC subsets in different clinical settings and different immunological compartments. Identification of better markers will facilitate these studies. More in-depth analysis of the interaction of MDSC with other cell types will help understanding the biological function and finally, the specific targeting of human MDSCs will enhance the effect of immune-based therapies in cancer.

Materials and methods: MDSCs have gained a lot of attention in recent years, mainly in mouse models. However, the results from murine studies indicate that human MDSCs will need to be analyzed in more detail in cancer patients in order to understand the induction and function of these cells in patients. One major hurdle remains the heterogeneity of these cells. humans, MDSC are inadequately characterized because of the lack of uniform markers. The described phenotype includes CD33+, CD11b HLA-DR- cells (immature phenotype) CD14+, HLA-DR- cells (monocytic like) and CD15+, HLA-DR- cells (granulocytic like). They negatively regulate the immune response by effecting NK and T cells. Potential mechanisms, which underlie this inhibitory activity range from those requiring direct cell-cellcontact. The only possibility to overcome this problem will be a careful phenotypical and functional analysis of all potential MDSC subsets in different clinical settings and different immunological compartments. Identification of better markers will facilitate these studies. More in-depth analysis of the interaction of MDSC with other cell types will help understanding the biological function and finally, the specific targeting of human MDSCs will enhance the effect of immune-based therapies in cancer.

Results: Here we show that human Hepatic Stellate Cells have the capability to induce Myeloid derived Suppressor Cells from circulation Monocytes in a cell-cell contact dependent mechanism.

Conclusions: Further investigation of the exact mechanism will provide opportunities to prevent the induction of Myeloid Derived Suppressor Cells. This represents a potential target for immunotherapy.

P0995

Induction of sustained tolerance towards experimental ConA hepatitis depends on CD4+ T and NKT cells

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Purpose/Objective: In mice, a single i.v. injection of the T cell mitogen Concanavalin A (ConA) leads to severe acute T cell and Kupffer Cell (KC) dependent hepatitis. Following a sublethal treatment of ConA, mice develop resistance towards hepatitis after additional

ConA injections within 8 days. This resistance is characterized by regulatory T cell and KC derived IL-10 dependent immunoregulation. However, IL-10 production was less regulated during sustained tolerance observed between 14 and 42 days. Here we investigated the role for B cells, $CD4^+$ T and NKT cells, Tregs and KCs in the induction of long-lasting liver tolerance during the first ConA treatment.

Materials and methods: Liver damage was quantified by plasma transaminase activities and/or by histology 8 h after a single ConA injection or after ConA restimulation 14 days after the first ConA treatment. Tregs were depleted from DEREG (DEpletion of REGulatory T cells) mice by diphtheria toxin i.p. treatment 1 day before first ConA injection. KCs were depleted by i.v. injection of Cl_2MDP liposomes 2 days before the first ConA stimulation. A role for antibody mediated protection was investigated by ConA restimulation in Jh/Jk double knock-out mice 42 days after the first ConA treatment. To investigate if induction of liver resistance depends on $CD4^+$ T cells, $RAG1^{-/-}$ mice were reconstituted with liver $CD4^+$ T cells and treated with ConA.

Results: Liver injury was detectable 8 h after a single ConA injection but was completely absent 14 days after ConA restimulation. However, neither absence of Tregs nor absence of KCs during the first ConA treatment abrogated liver tolerance in response to ConA restimulation on day 14. Also, B cell deficiency did not abrogate liver tolerance towards ConA. ConA treatment of $Rag1^{-/-}$ mice reconstituted with $CD4^+$ T cells induced hepatic injury. ConA tolerance upon restimulation was inducible in these reconstituted mice. However, ConA pretreatment of $RAG^{-/-}$ mice 14 days prior to reconstitution failed to induce tolerance.

Conclusions: $CD4^+$ T and NKT cells seem to be involved in induction of sustained liver tolerance towards ConA observed from day 14 onwards. Involvement of Tregs and KCs during the first ConA treatment might play a minor role in the induction of long-lasting liver tolerance towards ConA.

P0997

Innate and adaptive control of immunity to *Salmonella* in the liver

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Purpose/Objective: Non-typhoidal *Salmonella* e (NTS) such as *Salmonella* Typhimurium (STm) can cause invasive and often fatal disease in children and HIV-infected adults in developing countries. The liver is a major target of this infection yet how immunity is regulated in this site is incompletely understood. Whilst important for understanding how STm can kill, examining hepatic immune function also allows the study of how innate and adaptive responses co-ordinate to control infection in non-lymphoid sites.

Materials and methods: Microscopy, FACS and biochemical techniques were used to assess the impact of systemic STm infection on the murine liver in a resolving model of NTS infection.

Results: There was a profound and rapid impact of infection on the liver. Inflammatory lesions formed from day 2 of infection, peaked as T cells effected STm clearance and had resolved when bacteria had been cleared. Despite the striking inflammatory response and substantial necrosis, liver function was largely normal. Lesions mostly contained multiple myeloid $CD11c^+$ and $F4/80^+$ subsets, with small numbers of CD4 and CD8 T cells, but not B cells. Before infection, Kupffer cells were the dominant monocyte population but this changed rapidly after infection. T cells orchestrated foci development. In particular, T-bet in T cells was required for optimal T cell migration and T cell IFNγ, but not TNFα, expression. In addition, loss of T-bet increased numbers of hepatic $FoxP3^+$ T cells but did not alter the early inflammatory response. In contrast, infected IFNγ KO livers resembled those of non-infected mice despite having substantial bacterial burdens.

Conclusions: This work details the complex phenotype and organization of hepatic lesions after STm infection and the discrete roles of IFNγ in this response. Moreover, it identifies a central role for T-bet in balancing T cell phenotype in the liver post-infection. Exploiting these findings will help identify how best to promote bacterial clearance whilst minimizing the collateral cost of immune responses to the host.

P0998

LLT1 engages CD161 at the immunological synapse between T cells and hepatocytes

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Purpose/Objective: CD161+ CD8+ T cells are liver resident and associated with chronic hepatitis C virus infection. However, little is known about how CD161+ T cells engage target cells at a molecular level. CD161 is a co-stimulatory molecule that engages Lectin Like Transcript 1 (LLT1) on target cells and potentiates IFNγ production by T cells. It may thus be important in generating an effective anti-viral immune response. We have investigated the expression of LLT1 by hepatocytes, and the partitioning of CD161 and LLT1 at the immunological synapse between T cells and hepatocytes.

Materials and methods: LLT1 expression was investigated by flow cytometry and semi-quantitative RT-PCR in (1) Huh7 cells, (2) Huh7 cells containing the JFH-1 subgenomic replicon, and (3) Huh7/replicon cells cured with IFNα. CD161 and LLT-1 were fluorescently tagged and expressed in immortalized T cells (Jurkat) or Huh7 cells, and their distribution at the T cell/hepatocyte immune synapse determined by confocal microscopy.

Results: Huh7 cells constitutively expressed low levels of LLT1. The JFH-1 subgenomic replicon cells upregulated LLT1 at both surface protein and mRNA levels. Huh7 cells stimulated with TLR3 and TLR 7/8 agonists known to upregulate LLT1 on hematopoietic cells, did not show increased levels of LLT1. CD161 and LLT1 colocalised at T cell/hepatocyte immune synapses. These molecules formed microdomains which gradually coalesced into extended, perforated accumulations which represent a novel, atypical spatial arrangement for costimulatory molecules in T cells.

Conclusions: This study demonstrates that HCV may upregulate LLT1 on infected hepatocytes, independent of TLR-mediated recognition of virus. Hepatocytes expressing LLT1 form a stable immunological synapse with T cells expressing CD161, which forms independent of the presence of antigen. Within the synapse, CD161/LLT1 show a unique spatial distribution, and such patterning may underpin the molecular mechanism of how CD161 enhances T cell activation.

P0999

Loss of chemokine receptor CCR6 diminishes recruitment of IL-17-producing gamma/delta T cells to the liver leading to increased liver inflammation and fibrosis

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Purpose/Objective: The chemokine receptor CCR6 is known to be expressed on some T helper cells and $\gamma\delta$ T cells, monocyte derived dendritic cells and B cell subsets. It plays an important role in mucosal

immunity, but its role in liver disease is largely unknown. In this study we investigated its functional relevance in chronic liver disease and hepatic fibrosis.

Materials and methods: Wildtype (wt) and CCR6$^{-/-}$ mice were treated with the hepatotoxic agent carbon tetrachloride (CCl_4) thrice weekly over a period of 4 weeks. Liver damage, inflammation and fibrosis development were assessed by biochemical methods, histology, immunostaining, flow cytometry and qPCR. Human liver samples from patients with chronic liver disease ($n = 50$) were analysed by qPCR.

Results: CCR6 and its ligand CCL20 are strongly upregulated upon chronic liver injury in mice and in human patients with cirrhosis. CCR6$^{-/-}$ mice develop more severe hepatic fibrosis compared to wt mice when treated with CCl_4. They also show higher liver inflammation compared to wt mice, as indicated by increased overall infiltration of immune cells to the liver. We could not detect changes in infiltrating macrophages or composition of T helper cell subtypes between wt and CCR6-deficient mice, but expression of interleukin-17 (IL-17) was significantly reduced in livers of CCR6$^{-/-}$ mice. While wt mice showed an accumulation of IL-17-producing $\gamma\delta$ T cells in the liver upon CCl_4 treatment, this cell type was markedly reduced in livers of CCR6$^{-/-}$ mice. The adoptive transfer of wt $\gamma\delta$ T cells into CCR6$^{-/-}$ mice restored hepatic inflammation and fibrosis in the chronic injury model.

Conclusions: We propose a CCR6-dependent pathway for recruitment of IL-17-producing $\gamma\delta$ T cells in chronic liver injury, that protects the liver from excessive inflammation and fibrogenesis.

P1000
Metformin aggravates Con A induced liver injury

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Purpose/Objective: Several cases of liver injury related to hepatotoxicity of metformin, the most commonly prescribed oral antidiabetic medication, have been reported recently but mechanism of metformin induced liver injury remains unclear. We tested metformin hepatotoxicity and its effects in Con A induced hepatitis in mice.

Materials and methods: Con A hepatitis was induced in susceptible C57BL/6 and CBA mice, relatively resistant BALB/c mice as well as in CBA iNOS$^{-/-}$ mice. Liver enzymes, histology, mononuclear cell (MNC) infiltration, cytokine production, expression of Akt, NF-kB, p38, AMPK, apoptosis of MNCs and autophagy were analyzed.

Results: Single injection (400 mg/kg dissolved in saline, i.p) metformin did not induce liver damage, but the same dose of metformin significantly enhanced Con A (12 mg/kg dissolved in saline i.v) induced liver injury in BALB/c C57Bl/6 and CBA mice as evaluated by liver enzymes and histology. We found an increased level of TNF-α, IFN-γ in the sera and an increased number of TNF-α, IFN-γ and IL-17 producing CD4+ T cells, IFN-γ producing NK and IL-4 producing NKT cells, activated CD80$^+$ CD86+ IL-12 producing F4/80+ macrophages and CD11c+ dendritic cells (DCs) and aggressive B2 cells all of which are involved in disease process. Liver specific (CD4$^+$ CXCR3+ Tbet+IL-10+ and CD4$^+$ CD69$^+$ CD25-) T regulatory cells were downregulated by metformin. Metformin significantly increased expression of both Akt and NF-kB in the liver as well as influx of activated CD4$^+$ CD27+ cells, CD4+ and CD8+ CD62L$^-$ CCR7$^-$ effector memory cells.

We also noticed significantly increase of iNOS expression both in the liver and spleen by metformin. Deletion of iNOS attenuated both Con A induced disease and the effects of metformin: there was no difference in percentage of liver specific T regulatory cells, total number of liver infiltrated effector T cells, macrophages and DCs.

Conclusions: Metformin aggravates Con A induced liver injury by enhancing activation of immune cells in Akt/NF-kB dependent and p38 and AMPK independent manner affecting influx of effector and liver specific regulatory cells and NO production. These data suggest that in the inflamed liver metformin have deleterious effects.

P1001
MHC class I transfer from stellate cells to LSEC allows for enhanced immune surveillance in the liver

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Purpose/Objective: The liver is known for its unique immunological properties. Besides liver parenchymal cells, the hepatocytes, also non-parenchymal cells like dendritic cells (DC), liver-resident macrophages, the Kupffer cells, and liver sinusoidal endothelial cells (LSEC) interact with passenger leukocytes and modulate immune responses. However, little is known about the contribution of stellate cells, known to be responsible for contraction of the vessel diameter and storage of vitamin A.

Here, we addressed the question, if stellate cells contribute to immune surveillance by (cross-) presenting exogenous antigen to circulating naïve or effector CD8 T cells and the consequences of such antigen presentation during viral infection of the liver.

Materials and methods: We employed a transgenic mouse model with stellate cell-specific expression of the MHC class I molecule H2-K^b under the control of the GFAP-promoter (GFAP-K^b mouse). Mice were infected with 5×10^8 pfu of Adenovirus expressing the model antigen ovalbumin (AdOVA) followed by adoptive transfer of activated OT-I T cells which recognize SIINFEKL, a H2-K^b restricted peptide of ovalbumin. Stellate cells and liver sinusoidal endothelial cells were isolated as described previously.

Results: Using this model system, we found that H2-K^b-restricted CD8 T cells were stimulated *in vivo* to proliferate and express cytokines suggesting that stellate cells could directly interact with circulating naïve CD8 T cells.

Further, we found that stellate cells play a role in generating antiviral CD8 T cell response to viral infection of the liver.

However, a detailed characterization of the GFAP-K^b mouse revealed that stellate cells transferred MHC-I molecules to other hepatic cell populations like LSEC, DCs and Kupffer cells. We excluded erratic gene expression driven by GFAP-promoter in LSEC by RT-PCR.

Rather, we directly demonstrate transfer of H2-K^b molecules from stellate cells to H2-K^b-negative LSEC. Such molecule-transfer rendered LSEC capable of stimulating H2-K^b-restricted CD8 T cells in an antigen-specific fashion.

Conclusions: Our results provide insight into new mechanisms of how local immune regulation in the liver can be achieved, i.e. by transfer of MHC-I molecules from stellate cells to LSEC. This phenomenon may contribute to the unique immune functions of the liver.

P1002

Modification of a single lysine in a CYP2E1 epitope induces immune-mediated DILI in BALB/c mice

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Purpose/Objective: Key steps in the pathogenesis of immune-mediated drug-induced liver injury (Im-DILI) have not been identified. After receiving halogenated anesthetics, anti-seizure medications, antibiotics or non-steroidal anti-inflammatory drugs, susceptible patients develop Im-DILI thereby increasing their morbidity and often their mortality. In anesthetic Im-DILI patients, granulocytic hepatitis, trifluoroacetyl chloride (TFA) and IL-4-mediated cytochrome P4502E1 (CYP2E1) IgG4 antibodies support the diagnosis, while CYP2E1 epitopes responsible for the pathogenesis of Im-DILI are unknown. We previously demonstrated a CYP2E1 epitope [Gly^{113}-Leu^{133} (JHDN5)] containing a single lysine that was recognized by sera from anesthetic DILI patients with specific MHC II haplotypes. We showed that JHDN5 was recognized by splenocytes from mice with experimental Im-DILI induced by immunizations with liver proteins covalently altered by TFA, a drug hapten formed during metabolism of halogenated anesthetics. We hypothesize that covalent modification of a single lysine in JHDN5 induces IL-4-mediated, Im-DILI in BALB/c mice.
Materials and methods: JDN5 was modified by TFA (TFA-JHDN5) using the methods of Goldberger and Anfinisen. We confirmed 81.5% modification of JHDN5 using the method of Habeeb. BALB/c mice were immunized with 100 μg of an unrelated CYP2E1 epitope or JHDN5 ± TFA emulsified in CFA or CFA alone on days 0 and 7 and killed on day 21. IL-4 deficient (KO) mice were similarly treated with CFA ± TFA-JHDN5. Histology scores, antibodies and cytokine levels were analyzed using Mann−Whitney *U*-test. A P value <0.05 was significant.
Results: TFA-JHDN5 induced more granulocytic hepatitis ($P < 0.01$) as well as anti-TFA and anti-CYP2E1 antibodies ($P < 0.05$) than CFA-immunized BALB/c or KO mice. Granulocyte and macrophage attractants KC, MIP-2, G-CSF, M-CSF, MCP-1, MIP-1a, MIP-1b and VEGF as well as IL-7 and IL-9 were elevated in BALB/c but not KO livers ($P < 0.05$). Unmodified epitopes did not induce Im-DILI.
Conclusions: We confirm that covalent modification of a single lysine in a CYP2E1 epitope induces Im-DILI in BALB/c mice with features reminiscent of anesthetic Im-DILI in patients. Future studies of this epitope may uncover unidentified mechanisms of Im-DILI from other drugs and help to develop targeted agents to either treat or prevent this disease.

P1004

Overexpression of SMAD7 protects liver from TGFb/Smad-mediated fibrogenesis

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Purpose/Objective: SMAD7 is a negative regulator of TGFb/activin pathway. Recently, animal studies have shown that SMAD7 induction ameliorates TGFb/Smad-mediated fibrogenesis, suggesting a protective mechanism against liver injury. This study was scheduled to examine the role of SMAD7 in liver inflammation, fibrosis and the possible effect of antiviral treatment.
Materials and methods: Liver biopsies from 67 patients with hepatic diseases were studied: (1) 18 with chronic HCV hepatitis (CHC); (2) 19 with chronic HBV hepatitis at diagnosis (CHB/d); (3) four with CHB after antiviral treatment and relapse (CHB/non-r) (4) 14 with CHB after antiviral treatment response and remission for >5 years (CHB/r); (5) 12 with non alcoholic fatty liver disease (NAFLD). Three liver samples with a mild increase of aminotransferases but without histological changes, served as controls. Histological activity index and staging of fibrosis were also assessed. RNA was extracted and cDNA was synthesized using standard protocols. mRNA expression of TGFb isoforms (*TGFB1, 2, 3*), activins (*A, B, C, E*), *ALK4*, *ALK5*, SMAD molecules (*SMAD2, 3, 4, 7*), and *CTGF*was examined using quantitative real time PCR. Statistical analysis was performed using SPSS and P values < 0.05 were considered significant.
Results: Patients with CHB/r exhibited a significant increase of *SMAD7* and *ALK4* mRNA expression compared to CHB/d patients, and reduced levels of *TGFB1, SMAD2, SMAD3*, and *CTGF*. A significant increase of *SMAD7* was also found in NAFLD patients compared to untreated viral hepatitis patients and those who did not respond to any treatment. Moreover, NAFLD patients were presented with elevated levels of *TGFB1, TGFB3, INHβC, ALK5*, and *SMAD4.* Considering the intensity of inflammation, *SMAD7, ALK5*, and *INHβC* exhibited a significant increased expression from absent to minimal inflammation with a gradual reduction as inflammation exacerbates.
Conclusions: Our data indicate that in cases with low grade fibrosis, as NAFLD (characterized by a lower incidence of severe liver complications and fibrosis progression) and CHB/r, *SMAD7* overexpression might be a mechanism limiting the fibrogenic effect of TGFb suggesting that its induction may provide a target for novel therapeutic approaches.

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P1005

Peritoneal macrophage inflammatory profile in cirrhosis is dependent on the etiology and is related to ERK phosphorylation level

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Purpose/Objective: The aim of this work is to identify functional differences in the inflammatory profile of monocyte-derived macrophages (M-DM) from ascites in cirrhotic patients of different etiologies, alcohol- and hepatitis C virus (HCV)-related cirrhosis, trying to extrapolate studies from liver biopsies to immune cells in ascites.
Materials and methods: We studied 45 patients with cirrhosis and non-infected ascites, distributed according to disease etiology, HCV ($n = 15$) or alcohol ($n = 30$). Cytokines and cellular content in ascites were assessed by ELISA and flow cytometry, respectively. Cytokines and ERK phosphorylation level from peritoneal monocyte-derived macrophages isolated and stimulated *in vitro* were also determined.
Results: A different pattern of leukocyte migration to peritoneal cavity and primed status of macrophages in cirrhosis is observed depending on the viral or alcoholic etiology. Whereas no differences in peripheral

blood cell subpopulations could be achieved, T lymphocyte, monocyte and polymorphonuclear cell populations in ascites were more abundant in HCV versus alcohol etiology. Cirrhosis of HCV etiology is associated to a decreased inflammatory profile in ascites compared with alcoholic etiology. Higher levels of IL-10 and lower levels of IL-6 and IL-12 were present in ascitic fluid from HCV group. Isolated peritoneal monocyte-derived macrophages kept their primed status *in vitro* for the extent of 24 h culture. Increased phosphorylation of ERK1/2 was observed in ALC peritoneal macrophages at baseline compared with those from HCV patients, although addition of LPS induced higher phosphorylation increases of ERK1/2 in macrophages from HCV than from ALC patients.
Conclusions;An increased macrophage inflammatory status is present in ascites of alcohol-related cirrhotic patients compared with that of HCV-related. This fact could be related to differences in bacterial translocation episodies or regulatory T cell populations. These findings would contribute to identify potential prognostic and/or therapeutic targets for chronic liver diseases of different etiology.

P1006
Positive M2-ELISAs with negative anti-mitochondrial antibodies (AMA) on IFT: a diagnostic dilemma?

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Purpose/Objective: Immune fluorescence testing (IFT) combined with ELISA techniques to determine the specificity of autoantibodies have refined and facilitated the diagnosis of primary biliary cirrhosis (PBC) over the past years. Positive AMA with specificity for the M2 antigen have become an established diagnostic criterion. However, in our liver clinic we encountered a substantial number of patients with negative AMA who nevertheless showed positive ELISA results for M2 antibodies. The aim of the present study was to evaluate the significance of positive M2-ELISA- results with discordant negative IFT Results.
Materials and methods: Patient sera were screened using Hep2-cells (Inova), rat kidney, liver, and stomach sections (Menarini) and the M2 EP (MIT3)-ELISA (Inova). All sera screened for M2 antibodies in 2011 were included.
Results: Of 278 patients tested for M2 antibodies in 2011, 112 (40.3%) were positive. Surprisingly, 26 of these (23.2%) were judged negative for AMA on tissue sections. In nine of these cases (34.6%) PBC was diagnosed on the basis of clinical/histological criteria. In AMA/M2-discordant patients, ELISA Titers were significantly higher in patients suffering from PBC according to the guidelines (69.68 ± 41.22 units) than in ānon-PBC' patients (31.85 ± 8.332 units; $P = 0.0011$). Nine AMA/M2-discordant patients showed untypical cytoplasmic staining patterns in Hep2 cells, of whom 4 (44.4%) were diagnosed as having PBC. Of the 17 patients without cytoplasmic staining, 5 (29.4%) were diagnosed with PBC.
Conclusions: Some patients with PBC can present with negative testing for AMA, but react positive in the M2-ELISA. Conversely, patients with conditions other than PBC can exhibit positive M2-ELISAs. Most of these latter patients have elevated immunoglobulins on analysis, and M2-ELISA-levels in these patients are usually low. In conclusion, if clinical suspicion of PBC exists, M2 testing should be performed even in the absence of positive AMA.

P1008
Recruitment mechanisms for primary and malignant B cells to the human liver

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Purpose/Objective: B cells are present within chronically inflamed liver tissue and recent evidence implicates them in the progression of liver disease. In addition a large proportion of hepatic lymphomas are of B cell origin. The molecular signals that regulate normal and malignant B cell recruitment into peripheral tissue from blood are poorly understood leading us to study human B cell migration through hepatic sinusoidal endothelial cells (HSEC) in flow-based adhesion assays.
Materials and methods: We used isolated human HSEC in flow assays with purified peripheral blood B cells to elucidate the molecular mechanisms of B cell recruitment via HSEC. The contribution of conventional adhesion molecules, ICAM-1 and VCAM-1 and unconventional molecules VAP-1 and CLEVER-1/stabilin-1 was assessed by using function blocking antibodies. We repeated our experiments with two B cell lymphoma cell lines, CRL-2261 and Karpas 422, and primary malignant B cells. We assessed the contribution of chemokines by performing transwell assays and adding chemokines to our flow assays. We also tracked the motility of B cells and lymphoma cell lines on HSEC using tracking software.
Results: In flow assays B cells were captured from shear flow without a prior rolling phase and underwent firm adhesion mediated by VCAM-1. Unlike T cells, which displayed vigorous crawling behaviour on the endothelium, B cells remained static before a proportion underwent transendothelial migration mediated by a combination of ICAM-1, VAP-1, CLEVER-1/stabilin-1 and the chemokine receptor CXCR3 and CXCR4. B cell lymphoma cell lines and primary malignant B cells from patients with chronic lymphocytic leukaemia and marginal zone B cell lymphoma also underwent integrin-mediated firm adhesion involving ICAM-1 and/or VCAM-1 and demonstrated ICAM-1 dependent shape-change and crawling behaviour. Unlike primary lymphocytes the malignant cells did not undergo transendothelial migration which could explain why lymphomas are frequently characterised by intravascular accumulation of malignant cells in the hepatic sinusoids.
Conclusions: Our findings demonstrate that distinct combinations of signals promote B cell recruitment to the liver suggesting the possibility of novel targets to modulate liver inflammation in disease. Certain features of lymphocyte homing are maintained in lymphoma recruitment to the liver suggesting that therapeutic targets for lymphocyte recruitment may also prevent hepatic lymphoma dissemination.

P1009
Relationship between synthesis of alpha2-macroglobulin and changes in serum cytokine in rats

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Purpose/Objective: The α_2-macroglobulin (α2M) is a typical acute phase protein in rats. We have investigated the kinetics of α2M after inflammatory stimulation. Furthermore, we estimated that interleukin (IL)-6 and cytokine-induced neutrophil chemoattractant-1 (CINC-1) were contributed the synthesis of α2M. However, little is available on the time-dependent changes on synthesis of α2M in hepatocytes. In

this study, synthesis of α2M in hepatocytes was estimated by immunohistochemistry and correlations between synthesis of α2M and cytokines (IL-6, CINC-1) were investigated.
Materials and methods: Sprague-Dawley rats (age, 9 weeks) were used. Turpentine oil was intramuscularly injected at 0.4 ml/rat to induce acute inflammation. Three rats at each time point were scarified under anesthesia with pentobarbital before treatment and at 6, 12, 18, 24, 36, 48 or 72 h after injection of turpentine oil. Blood was collected from the aorta and the liver was removed. The presence of α2M in the liver was investigated by immunohistochemistry, and serum levels of α2M, IL-6 and CINC-1 were measured by ELISA.
Results: α2M was not detected in the liver before injection of turpentine oil. α2M was detected in whole lobules of liver after 12 h after injection of turpentine oil, when high serum levels of IL-6 or CINC-1 were observed. α2M was distributed around the central vein at 36 h. However, small amounts of α2M were detected in the liver at 48 h, when peak serum levels of α2M were observed. α2M was apparently synthesized in response to stimulation by IL-6 and CINC-1, and synthesis of α2M in hepatocytes peaked long before peak α2M serum levels were seen.
Conclusions: In conclusion, synthesis of α2M appears to be correlated closely with serum levels of IL-6 and CINC-1.

P1010

Role of mitogen activated protein kinases and PI3K-Akt on the cytokine inflammatory profile of peritoneal macrophages from ascites of cirrhotic patients

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Purpose/Objective: To compare the role played by several Mitogen Activated Protein Kinases (MAPKs) and PI3K-Akt pathways on the release of cytokines in monocyte-derived macrophages (M-DM) obtained from the ascites of cirrhotic patients to identify novel targets for pharmaceutical intervention to prevent hepatic damage.
Materials and methods: M-DM were isolated from ascites of cirrhotic patients and stimulated *in vitro* with LPS and heat killed *Candida albicans* in the presence or absence of the inhibitors for MEK1, p38 MAPK, JNK and PI3K. Ascites and cell culture supernatants were assayed by ELISA for TNF-a, IL-6 and IL-10. MAPK phosphorylation levels were determined by Western blot.
Results: We found that release of the pro-inflammatory cytokines, IL-6 and TNF-alpha at baseline was more effectively reduced by the MAPK inhibitors, while basal IL-10 anti-inflammatory cytokine secretion, was only, but strongly (91.6%) affected by inhibition of PI3K. The incubation of peritoneal M-DMs in the presence of LPS and heat killed *C. albicans* increased the release of IL-6, TNF-alpha and IL-10. LPS-induced pro-inflammatory secretion was more sensitive to MAK inhibitors, while that induced by *C. albicans* was more susceptible to inhibition of PI3K. Finally, inhibition of PI3K almost completely suppressed secretion of IL-10 in stimulated M-DM.
Conclusions: These results demonstrate pro-inflammatory cytokines release depends on MAPK signalling pathways and differ depending of microbial stimulus in this clinical setting, and confirm the prominent role of PI3K-Akt pathway in the modulation of IL-10 mediated anti-inflammatory function.

P1011

Salmonella infection promotes cross reaction of gut activated t cells to liver antigen leading to immune-mediated cholangitis in mice

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Purpose/Objective: A dysregulated immune response against components of the gut flora may be involved in the pathogenesis of inflammatory bowel diseases (IBD). Infections with pathogens could further trigger cross reactions against extraintestinal autoantigens in genetically susceptible individuals. Primary sclerosing cholangitis (PSC) is strongly associated with IBD. T cells infiltrating the livers of patients with PSC display an $\alpha 4\beta 7^+$ $CCR9^+$ phenotype, indicating their provenance from the gut-associated lymphatic tissues (GALT). This finding led to the hypothesis that activation of the adaptive immune system in the GALT could initiate an aberrant reaction against antigens in the liver.
Materials and methods: Naive antigen-specific CD8 OT-I T cells were transferred *i.v.* into transgenic mice, which express their nominal antigen ovalbumin (OVA) in enterocytes of the small intestine (iFABP-tOVA) or in the bile duct epithelia (ASBT-OVA), or into double transgenic mice (iFABPxASBT-OVA). To evaluate whether *Salmonella* promotes immune-mediated liver disease, mice were infected orally with *S. typhimurium* SL7207 (Sm) or *S. typhimurium* SL7207 expressing the MHC-I epitope $OVA_{257-264}$ (SmOVA). The effector function of OT-I T cells was evaluated by measuring IFN-g production and by an *in vivo* cytotoxicity assay. Inflammation in the liver was assessed by measuring plasma ALT and by histologic analysis.
Results: Adoptively transferred OT-I T cells acquired effector function characterized by production of IFN-γ and *in vivo* cytotoxicity in iFABP-tOVA as well as in double transgenic iFABPxASBT-OVA mice, while only a minority of OT-I T cells became activated in ASBT-OVA mice. Activated OT-I T cells migrated into the liver, but caused cholangitis only in the presence of OVA in the biliary epithelia. ALT-levels were elevated in the plasma of double transgenic iFABPxASBT-OVA mice, but not in the single transgenic lines. Infection with Sm or SmOVA enhanced the activation of OT-I T cells in the GALT and exacerbated cholangitis in iFABPxASBT-OVA mice.
Conclusions: The activation of T cells in the gut led to cross reactivity in the liver when the same antigen was expressed in both compartments. The severity of cholangitis was increased by oral *Salmonella* infection. Our findings suggest that cross reactivity of gut-derived T cells to endogenous antigens may be involved in the pathogenesis of PSC.

P1012

SepSecS-induced TH17-associated autoimmune hepatitis in mice

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Purpose/Objective: Autoimmune Hepatitis (AIH) is a chronic inflammatory liver disease of unknown pathogenesis; its characteristics include intrahepatic periportal lymphocytic infiltrates and circulating autoantibodies. Autoimmunity to the SepSecS molecule may be of pathogenetic relevance, since SLA/LP autoantibodies that recognize the SepSecS molecule are highly specific for AIH. Here we explored the role of SepSecS immunity in AIH.
Materials and methods: Mice were immunised with recombinant murine SepSecS protein in complete Freund's adjuvant (CFA).

Primary immune responses were analysed by *in vitro* re-stimulation of draining lymph node cells. Liver inflammation and cytokine response of liver infiltrating lymphocytes were determined.

Results: SepSecS-immunised C57BL/6 mice and IL-$10^{-/-}$ mice on C57BL/6 background developed SLA/LP autoantibodies. However, SepSecS-immunised C57BL/6 mice did not develop liver inflammation; in contrast, IL-$10^{-/-}$ mice manifested histological liver inflammation with periportal lymphocytic infiltrates, reminiscent of human AIH. In response to saline/CFA, IL-$10^{-/-}$ mice showed mild parenchymal liver inflammation of a granulomatous type, but not the characteristic periportal inflammation seen in response to SepSecS/CFA. Lymphocytes from SepSecS-immunised IL-$10^{-/-}$ mice, but not from C57BL/6 mice could transfer histological hepatitis both to C57BL/6 or IL-$10^{-/-}$ mice. In response to CD3 antibody stimulation *in vitro*, lymph node cells of IL$10^{-/-}$ mice secreted more IL-17 than those of C57BL/6 mice (1230 versus 460 pg/ml), indicating a general tendency of IL$10^{-/-}$ mice for T_H17 differentiation. In response to SepSecS re-stimulation, lymph node cells of IL-$10^{-/-}$ mice secreted considerably more IL-6 (480 versus 65 pg/ml) and IL-17 (550 versus 15 pg/ml) than lymph node cells of C57BL/6 mice. Accordingly, liver-infiltrating lymphocytes, notably CD4 T cells, isolated from SepSecS/CFA immunised IL-$10^{-/-}$ mice secreted significantly higher amounts of IL-17 (524 versus 72 pg/ml) and IFNγ (7590 versus 1234 pg/ml) in response to SepSecS re-stimulation, than IL-$10^{-/-}$ mice immunised with saline/CFA.

Conclusions: Immunity to SepSecS can cause hepatitis in susceptible IL-$10^{-/-}$ mice. Periportal liver inflammation was associated with a T_H17 response of SepSecS-primed lymphocytes.

P1013

Serum HLA-G is associated with liver damage rather than with liver graft acceptance

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Purpose/Objective: HLA-G is a non-classical HLA class I molecule which expression in healthy individuals is restricted to invading trophoblasts in placenta, which is thought to contribute to protection of the fetus from immunological attack by the mother. On basis of cross-sectional studies it has been suggested that increased serum HLA-G levels after liver transplantation (LTX) are associated with graft acceptance. The aim of this study was to determine whether longitudinal variations in serum HLA-G concentrations after LTx are associated with signs of graft acceptance.

Materials and methods: Serum HLA-G levels were quantified by ELISA in a cohort of 32 patients with end-stage liver diseases, both before transplantation and at several time points during the first year after transplantation, and for comparison in 24 age- and gender-matched healthy subjects. In addition, HLA-G was quantified in T-tube bile collected during the first few weeks after LTx.

Results: Pre-LTX serum HLA-G levels in the patients were significantly higher compared to those in healthy individuals, suggesting that soluble HLA-G is released from diseased livers. Pre- and post-LTx serum HLA-G levels were positively correlated with serum transaminases and bilirubin, indicating release during liver damage. Patients with an early acute rejection episode displayed significantly elevated serum HLA-G concentrations compared to non-rejectors during the first 2 weeks after LTx. Starting at 1 month after LTX, serum HLA-G levels gradually decreased. Interestingly, bile secreted by the liver graft during the first few weeks after LTx contained HLA-G, suggesting that HLA-G is produced in the liver graft early after LTx.

Conclusions: Our data do not support the hypothesis of a tolerogenic role for HLA-G after LTX, but rather suggest that serum HLA-G levels are associated with liver (graft) damage.

P1014

Synergistic effect of HLA-class II DRB1*1301 and activating full length KIR2DS4 in the susceptibility to type I autoimmune hepatitis

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Purpose/Objective: A previous study from our center revealed a difference in the genetic predisposition to type I autoimmune hepatitis (AH) between children (PAH) and adults (AAH). The haplotype HLA-DRB1*1301-DQB1*603 is strongly associated with PAH in most pediatric populations studied worldwide. Because some activating KIR genes were associated with autoimmune diseases, in the present study we investigated the possibility of a combined effect of these two highly polymorphic systems.

Materials and methods: The study included 233 healthy controls, 81 type I PAH and 44 AAH patients. Genomic DNA was used to identify the presence or absence of each 16 KIR genes, by two PCR amplifications: PCR-1 for domains D1and D2 combined and the PCR-2 for the TM/cytoplasmic region. Nineteen 5«-digoxigenin-labeled probes were used in a sequence-specific oligo-nucleotide probing (SSOP) approach. The KIR2DS4 gene was amplified by PCR with primers to analize the full-length and the deleted version of KIR2DS4. HLA class I and class II alleles were typed by PCR amplification and hybridization with SSOP for the analysis of exon 2 and 3 (class I), or exon 2 polymorphisms (class II).

Results: The frequency of all 16 tested KIR genes were similar when we compared controls with AAH or PAH patients. In Argentinian Caucasoid healthy population, the frequency of KIR2DS4-alleles containing the 22 bp deletion in exon 5 account for 81% versus a frequency of the full-length gene of 39%. In contrast, in PAH patients the full-length 2DS4*001 allotype was present in 68% ($P < 0.0001$) and in 53% of AAH. We followed the method of Svejgaard and Ryder to find out whether class II allele or KIR full-length allele represent the primary association. The combined presence of both factors provided an OR value of 40.3, higher than the product of the RR of the two independent factors. The presence of the truncated form of KIR2DS4, showed a protector effect with an OR of 0.22. In AAH, only the combined presence of HLA-DRB1*1301 and functional KIR2DS4 showed an increased susceptibility (O $r = 5.8$) not observed by these two factors independently.

Conclusions: These results represent the first evidence of a synergistic effect between a class II allele and an activator KIR gene in the susceptibility to develop autoimmune hepatitis.

P1015

The ABCs of viral hepatitis – defining biomarker signatures for acute viral hepatitis

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Purpose/Objective: Viral hepatitis is the leading cause of liver disease worldwide and can be caused by several agents, including: Hepatitis A, B and C. The host response to liver infection has been an area of intense study. We exploited recent advances in the assessment of biomarker signatures in order to define unique and common responses during three acute infections, all sharing the same tissue tropism. Patients were recruited as part of a hospital based surveillance program in two 'fever hospitals' specialized in infectious diseases in Cairo, Egypt.

Materials and methods: We performed multi-analyte profiling (MAP) measuring the concentrations of 182 molecules in the serum of acute Hepatitis A, Hepatitis B, and Hepatitis C infected individuals, as well as healthy controls. Patients with negative anti-HCV Ab and positive HCV-RNA were considered as acute hepatitis C cases. Acute Hepatitis B was defined by a positive IgM anti-hepatitis B virus core and circulating levels of hepatitis B surface antigen. Acute Hepatitis A was defined as positive anti-HAV IgM Ab.

Results: Statistical analysis revealed an analyte signature based upon eight proteins, which applied to Principle Component Analysis distinguished HCV patients from HAV/HBV-infected individuals and healthy controls. Notably, the signatures of HAV and HBV host response were indistinguishable in a hierarchical cluster analysis of all samples; suggesting that these RNA and DNA viruses share a similar mode of pathology in contrast with Hepatitis C. However when HAV and HBV patients were directly compared, six differentially expressed serum proteins were identified. Within the Hepatitis C cohort we could separate cleared and non-cleared patients based on just five molecules. One of these was IP-10, which we and others have previously highlighted as a predictive biomarker of Hepatitis C clearance, thus validating the approach used here.

Conclusions: This medium throughput discovery approach has revealed previously unrecognized virus host interactions. The identification of hepatitis virus specific biomarkers will lead to novel functional insights of disease mechanisms.

P1016

The lymphotoxin-ß receptor and its role in hepatocyte-mediated liver regeneration

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Purpose/Objective: The liver retains a capacity for regeneration in response to injury. Loss of at least 30% of liver mass leads to synchronized proliferation of mature hepatocytes (compensatory hyperplasia). It has previously been shown that mice deficient in LTβR (LTβR$^{-/-}$) exhibit reduced survival after partial (70%) hepatectomy (PHx). Therefore, liver regeneration was analyzed in LTβR$^{-/-}$ mice compared to wild-type (WT) mice.

Materials and methods: 70% PHx was performed in WT and LTβR$^{-/-}$ (KO) mice. Using microarray (MA) analysis, the gene expression profile of liver tissue was analyzed 12 h post PHx and, where appropriate, verified by realtime RT-PCR. H/E-staining of liver sections and cytokine ELISAs were performed 0, 12, 24, and 48 h post PHx. Serum protein levels (GPT, GOT, Bilirubin, pancreatic amylase, alkaline phosphatase, glucose) were analyzed 0, 12, 24, and 48 h post PHx. At similar time points H/E-staining of liver sections and cytokine ELISAs were performed.

Results: It was confirmed that LTβR-deficient mice have a decreased survival rate compared to WT mice (62% versus 90%, respectively). Interestingly, surviving LTβR$^{-/-}$ animals show no delay in liver regeneration compared to WT animals. MA analysis identified a panel of differentially expressed genes; most prominent among these was a markedly decreased expression (in LTβR$^{-/-}$ mice) of murinoglobulin-2, a proteinase inhibitor of the α2-macroglobulin family. Also, TNF expression was increased twofold in the LTβR$^{-/-}$ cohort (confirmed by ELISA). Levels of the liver transaminases GPT and GOT were similar in both cohorts, while levels of pancreatic amylase were significantly decreased in LTβR$^{-/-}$ animals 24 h post PHx. In contrast, alkaline phosphatase was significantly increased in KO animals 24 and 48 h post PHx, and surprisingly, still elevated 10 days after PHx. Liver sections of LTβR$^{-/-}$ animals showed a higher number of hemorrhagic/necrotic areas and more vacuolisation of hepatocytes 24 and 48 h post PHx.

Conclusions: After PHx, LTβR$^{-/-}$ mice show a massive change in their gene expression profile compared to WT animals. In addition, their cytokine expression profile is altered and several serum proteins appear to be deregulated. This clearly demonstrates the importance of LTβR signaling in liver regeneration and its exact role in will be elucidated in further studies.

P1017

The role of Foxp3+ regulatory T cells in the development of an adaptive immune response in a persistent HBV mouse model

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Purpose/Objective: Worldwide around 2 billion people are infected with the Hepatitis B Virus (HBV), from whom approximately 350 million people are chronically infected. They are at high risk of developing liver cirrhosis and hepatocellular carcinoma (HCC). To facilitate the development of therapeutic strategies against chronic Hepatitis B infection, it is mandatory to have a better understanding of the detailed mechanism responsible for the induction of HBV persistence.

The forkhead box P3 transcription factor (Foxp3) has been shown to influence several adaptive immune responses, for example in the context of LCMV, where protective roles for regulatory T cells (Treg) have been uncovered (Rowe *et al.*, Immunology 2012). Here we analyze the role of Foxp3$^+$ Treg on the induction of HBV-specific CTL responses upon HBV infection in a novel mouse model.

Materials and methods: As *in vivo* studies of HBV are hampered by the lack of a suitable animal model, we here take advantage of our recently developed mouse model for the induction of HBV persistence in mice (Huang *et al.*, Gastroenterology 2012).

These mice were infected with a low-dose (1×10^8 i.u./mouse) adenoviral vector carrying a 1.3-fold overlength human HBV genome (AdHBV) to cross the species barrier.

To deplete Foxp3$^+$ Treg, we used Foxp3.LuciDTR-4, which display 95% Treg depletion following injection of diphtheria toxin (DT) (Suffner *et al.*, J. Immunology 2009).

Results: In AdHBV infected Foxp3.LuciDTR-4 mice we observed a dramatic decline of the serum HBsAg but not HBeAg level, which dropped below detection limit at d10, whereas the control groups remained positive for HBsAg. Additionally, the serum ALT level of those mice peaked at day 7 by fourfold. Further, we analyzed the HBV-specific CTL response among total CD8$^+$ T cells. We found that after

depleting Foxp3$^+$ cells there is an increase in HBs$_{190-97}$ -specific, but not HBc$_{93-100}$ -specific CD8$^+$ CTLs in the liver and spleen.
Conclusions: During persistent HBV infection, Foxp3$^+$ Treg plays a role in the suppression of HBV-specific CTL response, especially for the development of an HBs-specific adaptive immunity. Further analysis for revealing the detailed mechanism of tolerance induction towards HBV has to be investigated.

P1018

The secretion of cytokines has a greater influence on the severity of acute hepatitis than the survival of infiltrating CD8 T cells

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Purpose/Objective: Acute hepatitis is often mediated by CD8 T cells that kill target cells or secrete hepatotoxic cytokines. Although it is known how cytotoxic T cells mediate liver damage, the parameters that regulate acute hepatitis are not well understood. To determine whether some intrinsic T cell parameters might play a role in this regulation, we used the well-characterized Met-K^{b}Tg mouse model of autoimmune mediated hepatitis (AIH).
Materials and methods: Acute liver injury in Met-K^b mice is induced by the adoptive transfer of TCR Tg CD8 T cells activated in lymph nodes and recognizing their cognate antigen in the liver. Tg CD8 T cells transferred into Met-K^b typically induced a severe but self-limiting autoimmune hepatitis (AIH). To investigate whether cytokine regulation and apoptosis of CD8 T cells were critical in limiting the acute damage induced by CTLs, we performed transfer experiments using TCR transgenic T cells deficient for either suppressor of cytokine signaling (SOCS-1) or the pro-apoptotic molecule Bim.
Results: Bim$^{-/-}$ Tg cells accumulated in liver of recipient mice at sixfold higher levels compared to wt Tg cells. Despite this substantial accumulation, the outcome of hepatitis remained unchanged, neither prolonged nor more severe. This data suggest that although T cells died in a Bim-dependent process, T cell survival was not a critical parameter in limiting liver damage.

In contrast, SOCS-1-deficient Tg T cells induced a more severe hepatitis than their wt counterparts. SOCS-1$^{-/-}$ Tg isolated from the liver displayed upregulated IL-2Ra chain, required to form the high affinity IL-2R. Upregulated IL-2Rawas associated with higher IFNg, CTL activity, and proliferation rates, consistent with enhanced effector function and supporting a critical role for cytokines in CTL function.
Conclusions: These data demonstrate that the propensity of CD8 T cells to mediate acute hepatitis is determined by the quality, rather than the quantity of CTLs infiltrating the liver. In the long term, both Bim and SOCS deficiency led to the accumulation of CD8 T cells that were unable to cause chronic liver damage. These findings reveal the existence of mechanisms that are able to silence potentially autoreactive T cells surviving acute hepatitis. Such mechanisms might explain why immune infiltrates do not always correlate with ALT levels in patients chronically infected with HCV or with autoimmune hepatitis.

P1019

TREM-1 expression is elevated on monocytes in cirrhotic patients

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Purpose/Objective: Sepsis and spontaneous bacterial peritonitis (SBP) are common sequelae in patients with cirrhosis. There is increasing interest in the role of monocytes in bacterial infection in cirrhotics. Triggering Receptor Expressed on Myelocytes 1 (TREM-1) modulates the immune response via an intracellular signalling cascade with resultant production of pro-inflammatory cytokines. It has been used as a biomarker in the diagnosis of bacterial infection but its role in patients with cirrhosis is unknown. We aim to evaluate TREM-1 as a biomarker in the diagnosis of SBP.
Materials and methods: Venous blood samples were obtained from 11 healthy controls (HC) and 14 patients with advanced cirrhosis (CA), as defined by clinico-radiological criteria. Simultaneous ascitic fluid samples were taken from seven patients in the CA group. Cirrhosis severity was graded using Child-Pugh score (median: 10) and Model for End-Stage Liver Disease score (MELD) (median: 14). The CA group had no clinical or laboratory evidence of sepsis at the time of sampling. Flow cytometry was used to quantify the expression of TREM-1 on three monocyte subsets: CD14$^+$CD16-, CD14$^+$CD16+, CD14dimCD16+ and CD16$^+$CD15+ neutrophils in blood and CD14+ monocytes and CD16$^+$CD15+ neutrophils in ascitic fluid.
Results: Results to date show that TREM-1 expression is significantly higher in the CA group compared to HC in the monocyte subsets CD14$^+$ CD16- [median geometric mean fluorescent intensity (GMFI): 6250 versus 2663 ($P = 0.0067$)] and CD14$^+$CD16$^+$ [GMFI: 4666 versus 9280 ($P = 0.0148$)] but not CD14dimCD16$^+$ or CD16$^+$ CD15$^+$ neutrophils. There is no correlation between TREM-1 expression and severity of cirrhosis by Child-Pugh or MELD score. There is no significant difference in TREM-1 expression on monocytes in ascitic fluid compared with CD14$^+$ CD16$^+$ monocytes in blood.
Conclusions: Blood monocyte TREM-1 levels are elevated in the CA group compared to the HC group in the absence of infection. There was no difference in TREM-1 expression between blood and ascitic fluid monocytes in culture negative and non-neutrocytic ascites (PMN count <250 cells/mm^3). On-going studies are evaluating the use of a biomarker panel comprising TREM-1, CD11b, CCR2 and CD64 in the diagnosis of SBP.

P1020

Tumor necrosis factor plays a vital role in mediating acetaminophen hepatotoxicity

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Purpose/Objective: Acetaminophen (APAP) is a widely used analgesic, and overdose of APAP may cause acute liver failure. APAP induces direct hepatotoxicity, and the necrotic hepatocytes further trigger an acute inflammatory response in the liver. Whether the inflammatory response and recruited leukocytes contribute to APAP-induced liver injury is still controversial. In this study, we investigated the role of the pleiotropic proinflammatory cytokine tumor necrosis factor (TNF) in APAP-induced liver injury.
Materials and methods: Overdoes of APAP (300 mg/kg) was administered to C57BL/6, TNF$^{-/-}$ and TNFR1$^{-/-}$ mice intraperitoneally, and serum levels of ALT, TNF, IL-6, and IL-1β and liver myeloperoxidase

activity were determined at various times after APAP challenge. To evaluate the effect of pharmacological TNF blockade, mice were given etanercept (Enbrel®) prior to APAP challenge.
Results: APAP-induced liver injury and hepatitis were markedly attenuated in $TNFR1^{-/-}$ and $TNF^{-/-}$ mice, and wild-type animals treated with the TNF blocker etanercept were also protected from APAP hepatotoxicity, indicating that TNF plays an important role in mediating AAP-induced hepatitis. Using the bone marrow chimeric mouse model, we further identified that TNFR1 function was required on radiosensitive non-parenchymal cells.
Conclusions: The production of TNF and its action on the TNFR1 of non-parenchymal cells play a vital role in mediating APAP hepatotoxicity.

P1021

Xenobiotics induced mouse model of human primary biliary cirrhosis develop PDC-E2 specific autoreactive T and B cells

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Purpose/Objective: Primary biliary cirrhosis (PBC) is a liver specific autoimmune disease characterized by antimitochondrial autoantibodies (AMAs), infiltration of lymphocytes in portal tracts and progressive destruction of intrahepatic bile duct. The major autoantigen in human PBC is the E2 subunits of the pyruvate dehydrogenase complex (PDC-E2). Increased serum levels of anti-PDC-E2 antibodies and liver T cells against PDC-E2 have been observed in patients with PBC, suggesting that autoreactive T cells and B cells play roles in the pathogenesis of PBC. We have developed the mouse PBC based upon immunization with xenobiotics 2-OA-BSA and a-galactosylceramide (a-GalCer), an invariant natural killer T cell activator. Such mice, similar to human PBC, develop high titer AMAs, autoimmune cholangitis and fibrosis. However, there has not been attempted at defining the presence of autoreactive T cells and B cells and, in particular, whether such cells are directed against PDC-E2.
Materials and methods: To address this issue, T cells and B cells against PDC-E2 from 2-OA-BSA/a-GalCer immunized mice were analyzed. For autoreactive T cells, liver mononuclear cells were stimulated with recombinant mouse PDC-E2 (rmPDC-E2) and cytokine production and proliferation of the antigen-stimulated T cells were examined. For autoreactive B cells, splenocytes were stimulated with rmPDC-E2 and anti-PDC-E2 antibodies in the culture supernatants were examined.
Results: Our results showed that there was no detection of reactive T and B cells in naïve mice when either control protein or rmPDC-E2 was used as antigens. Moreover, there was low detection of reactive T cells and B cells in 2-OA-BSA/a-GalCer immunized mice when cells were stimulated with no protein or control protein. However, significant increases of IFN-g and IL-17 production and proliferation of T cells as well as supernatant anti-PDC-E2 antibodies in rmPDC-E2 stimulated B cells in 2-OA-BSA/a-GalCer immunized mice were noted.
Conclusions: These data highlight not only the relevance of 2-OA-BSA/a-GalCer immunized mice as a model of human PBC, but also further imply that PDC-E2 is a major autoantigen and likely to be the antigenic driver of this disease.

Poster Session: HIV

P1022

Anti-retroviral drugs used in the management of HIV down-regulate PPAR-gamma binding, adipokine production and triglyceride storage in 3T3-L1 adipocytes

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Purpose/Objective: Anti-retroviral therapy (ART), used in the management of HIV, is associated with a lipodystrophy syndrome characterised by peripheral fat wasting, central obesity, dyslipidaemia, insulin resistance, inflammation and endothelial dysfunction. Peroxisome proliferator-activated receptor-γ (PPAR-γ) binding and downstream signalling have been shown to be down-regulated by ART. We hypothesise that anti-retroviral drugs decrease PPAR-γ binding to its gene response element, decrease adiponectin, increase leptin and resistin, and decrease triglyceride storage.
Materials and methods: 3T3-L1 pre-adipocytes were differentiated in the presence of zidovudine (1 μM), stavudine (10 μM), tenofovir (1 μM), ritonavir (20 μM) and indinavir (10 μM). We investigated triglyceride storage using Oil Red O staining, gene expression using real-time RT-PCR, protein secretion using a multiplex ELISA and PPAR-γ binding using a DNA-binding ELISA. Statistical analysis was performed using ANOVA (SPSS, v15).
Results: Triglyceride storage was significantly reduced in adipocytes treated with ritonavir relative to the vehicle (ethanol) control (29%; $P = 0.001$). Ritonavir significantly decreased PPAR-γ binding and gene expression relative to vehicle control (39%; $P = 0.03$ and 51%; $P = 0.004$, respectively). Ritonavir significantly decreased secretion of PPAR-γ target gene product, the anti-inflammatory cytokine adiponectin (97%; $P < 0.001$). Indinavir and tenofovir significantly decreased adiponectin secretion relative to vehicle control (21%; $P = 0.001$ and 30%; $P < 0.001$, respectively). Significant increases in expression of pro-inflammatory resistin ($P = 0.001$), leptin ($P < 0.001$) and IL-6 genes ($P < 0.001$) were caused by tenofovir, in leptin by zidovudine ($P = 0.005$) and in IL-6 by indinavir ($P = 0.017$).
Conclusions: These data indicate the detrimental effect of ART on triglyceride storage, PPAR-γ signalling, adiponectin secretion and expression of pro-inflammatory genes. Ongoing work is investigating whether long-chain polyunsaturated fatty acids, as activating ligands for PPAR-γ, can mitigate the effects of ART on adipocyte function, gene expression and inflammation by up-regulating PPAR-γ and adiponectin and down-regulating resistin, leptin and IL-6.

P1023

Association between HLA-B*40 and lipodystrophy in a population of HIV-infected patients from South of Spain

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Purpose/Objective: Some HIV-infected patients undergoing highly active antirretroviral therapy (HAART) develop adipose tissue redistribution and metabolic abnormalities called lipodystrophy syndrome (LD). The frecuency of lipodystrophy is approximately between 35–50% and host genetic factors might confer particulary susceptibility. The aim of this study was to determine the clinical risk factors and HLA-B* alleles associated with stavudine or zidovudine-associated lipodystrophy.
Materials and methods: A case-control study was conducted for HIV-patients receiving stavudine or zidovudine containing antirretroviral regimens. Clinical assessments for lipodystrophy by phisical examination and DEXA (dual-energy X-ray absorptiometry) were obtained. The patients were calssified into 2 groups: patients with lipodystrophy and patients without lipodystrophy (control group). We compared the HLA-B* allelic frequency between the case and the control group to determine the possible association with stavudine or zidovudine associates lipodystrophy.

DNA was isolated from anticoagulated peripheral blood mononuclear cells using standard techniques. The locus B HLA class I typing was made for all subjects by Luminex PCR-SSO (One Lambda). The data were analyzed using the chi-square test or Fisher exact test, the magnitude of associations was estimated by odds ratio by the Woolf-Haldane method.
Results: We studied 127 patients with HIV infection diagnosed in the infectious Diseases Service of Carlos Haya University Hospital in Malaga, south of Spain. Seventy three of them had lipodystrophy and fifty four were considered as controls. Frequencies distribution analysis of the alleles at the B locus showed a statistically significant increase in the frequency of HLA-B*40 in the group of patients with LD compared to those with no LD (13.6% versus 1.8%) $P = 0.01$.
Conclusions: Although greater numbers of patients are needed the HLA-B*40 allele seems confer susceptibility for lipodystrophy in patients with HIV in a population from south of Spain.

P1024

Atopic-like dermatitis as expression of post-kala-azar dermal diffuse leishmaniasis in HIV infection

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Purpose/Objective: To describe the case of post-kala-azar dermal diffuse leishmaniasis in HIV infection.
Materials and methods: A 52 yo. man with B3 HIV infection from 10 years, came for a 3-week history of dyspnoea, cough and diffuse itch. He was submitted 7 years before to splenectomy for a visceral leshamniosis (VL), treated by antimony therapy. He was suffering from hyperthyroidism, hyperuricemia and dilatative cardiomyopathy. He received multidrug treatment for the HIV infection, and specific therapies for the concomitant diseases. At the physical examination hepatomegaly and diffuse severe xerosis with fine scales on the face and on the body, especially on the flexor surfaces, were observed. Routine blood and urine tests, lymphocyte typing, Ig E serum level, stool cultures were requested. Although afebrile, he was submitted to pulmonary TC scan and sputum cultures for mycobacteria were performed. Skin biopsies for histologic examination and PCR analysis were obtained.
Results: The lab tests revealed severe anemia (Hgb 7.5 g/dl) with macrocytosis, lymphocytopenia (0.82×10^9/l; 11.7%), CD3 641 ml, CD4 327 ml, CD4/CD8 ratio of 1.08. Ig E level was normal and stool cultures negative. The CT showed signs of pneumonitis but sputum cultures did not reveal mycobacteria. Skin histology showed diffuse dermal mucinosis and oedema, massive infiltrate of lymphocytes, histiocytes fulfilled by intracytoplasmatic parasites. The bone marrow biopsy showed severe infiltration by amastigotes. PCR confirmed a Leshamania Donovani infection. Liposomal amphotericine B (5 mg/kg), given as a five-day course, induced 100% cure rate and was tolerated.

Conclusions: Post-kala-azar dermal leishmaniasis (PKDL) is a complication of VL in a patient who has recovered from visceral form and who is otherwise well. There is increasing evidence that the pathogenesis is largely immunologically mediated. Increased interleukin 10 in the peripheral blood of VL patients predict the development of PKDL. During VL, interferon γ is not produced by peripheral blood mononuclear cells (PBMC). After treatment of VL, PBMC start producing interferon γ, which coincides with the appearance of PKDL lesions? Interferon-γ-producing cells cause skin inflammation, as observed in classical atopic lesions, as a reaction to persisting parasites in the skin.

P1026

Cellular immune responses after a dendritic cells (DC)-based therapeutic vaccine in cART treated chronic hiv-infected patients

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Purpose/Objective: We have performed a blinded placebo-controlled study immunizing antiretroviral (cART) treated chronically HIV-1 infected patients with autologous Myeloid-Derived Dendritic Cells (MD-DCs) pulsed with heat inactivated autologous HIV-1.

Materials and methods: Of the 36 patients with $CD4^+$ >450 cells/mm^3 were randomized (2:1) to receive three immunizations every 2 weeks with DC pulsed with autologous heat-inactivated HIV-1 (Cases, $n = 24$) or with non-pulsed DC (Controls, $n = 12$). Changes in viral load (VL) as well as changes in CD4 cell counts have been evaluated. Additionally HIV specific responses were measured in PBMC samples from different time-points by LPR and by IFN-g-Elispot against gag, nef and gp41 HIV overlapping peptide pools, respectively.

Results: VL rebounded to detectable level in all the patients. At week 12 and 24, a decrease of VL ≥ 1 log was observed in 12/22 (55%) versus 1/11 (9%) and in 7/20 (35%) versus 0/10 (0%) in cases and controls, respectively ($P = 0.02$, $P = 0.03$). CD4 drop to baseline value before any cART without differences between groups. Although only transient positive responses to HIV p24 were observed, the median change in LPR to HIV p24 at week 24 from baseline was 0.96 versus -0.50 ($P = 0.02$) in cases versus controls, respectively. Baseline median values of the total sum of HIV specific responses against HIV peptide pools were similar in both arms (2625 versus 2283 SFC/10^6 PBMC, $P = 0.462$). After vaccination, the median change of total sum of SFC/10^6 PBMC at week 24 was 3567 versus 838 SFC/10^6 PBMC ($P = 0.0459$) in cases and controls, respectively. This difference was more evident when analyzing responses against gag p17 and Nef peptide pools ($P = 0.0288$ and $P = 0.03615$, respectively). No statistically significant correlations between immune responses and VL were found.

Conclusions: These results indicate that HIV-1 specific immune responses elicited by therapeutic DC vaccines could significantly change pVL set-point after cART interruption in chronic HIV-1 infected patients.

P1027

Chemokines CCL3, CCL4 and chemokine receptor CCR5 in HIV-1-infected women with complicated anamnesis

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Purpose/Objective: Chemokine receptor CCR5 plays a key role inhuman immunodeficiency virus (HIV) entry into CD4+ T lymphocytes. Chemokines CCL3 and CCL4 have been shown to possess antiviral effects by binding to the HIV-1 co-receptor CCR5. We evaluated activity of these mechanisms in HIV-infected women that used drugs, alcohol and additionally had sexual transmitted diseases.

Materials and methods: Eighty three HIV-1-infected women were divided in four groups: drug edicts (DE) — 20, prostitutes (P) — 21, sexual transmitted diseases (STD) — 23, chronic alcoholism (CA) — 19. Non-infected 30 women with chronic alcoholism and 30 women with acute alcohol intoxication served as control groups. Lymphocyte subsets and expression of CCR5 on CD4+ T lymphocytes were examined by flow cytometry. CCL3, CCL4, IL-8 in blood serum were detected by ELISA.

Results: Levels of CD4+ T lymphocytes, especially CD4/CD8 ratios were decreased dramatically in four groups of HIV+ women. Expression of CCR5 inCD4+ lymphocytes was decreased in all groups of HIV+ women too. Levels of CCL4 in groups DE, P and STD were decreased, but not in CA group. Changes in CCL3 were not significant in all groups of HIV+ women. Levels of IL-8 were elevated in all groups ofHIV+ women. In non-infected women with acute alcohol intoxication levels of CCL4 were decreased.

Conclusions: Drugs using and sexual transmitted diseases in HIV-1-infected women are related with suppressed CCR5 expression in CD4+ lymphocytes and levels of CCL4 in blood serum. Chronic alcoholism has not so great suppressive influence. Acute alcohol intoxication relates with lower levels of CCL4 in non-infected women and one should think about weaker resistance of organism against HIV in such women.

P1028

Co-infection of human immunodeficiency virus and hepatitis B surface antigen among pregnant women in Kaduna, Nigeria

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Purpose/Objective: Human immunodeficiency virus (HIV) and Hepatitis B virus (HBV) share overlapping transmission routes and some risk factors. The HIV-infected pregnant cohort represents a unique population and infection with HBV is considered a public health problem worldwide. This study was conducted to determine the sero-prevalence of HIV and Hepatitis B Surface Antigen (HBsAg) and co-infection in pregnant women in Kaduna, Nigeria.

Materials and methods: Eight hundred pregnant women attending ante-natal care in four selected hospitals were recruited for the study. Blood samples were collected and examined for the presence of HIV and HBsAg using test kits. The positive HIV blood samples were analyzed for $CD4^+$ counts, while the positive HBsAg plasma samples were confirmed using ELISA and tested for various markers of HBV.

Results: Human immunodeficiency virus was detected in 5.9% (47/800) and HBsAg was detected in 3.9% (31/800) of the blood samples of the pregnant women. Four (0.5%) of the women were co-infected with both viruses. Mean $CD4^+$ count in the HIV positive pregnant women

was 396 cells/μl of blood while the mean $CD4^+$ count in HIV and HBsAg co-infected women was 299 cells/μl of blood. Test for markers of HBV indicated Anti-HBc as the most predominant (58.1%:18/31) while Anti-HBs was the least (3.2%:1/31). The highest prevalence of 13% (7/54) and 4.4% (10/229) were recorded for HIV and HBsAg among women in age groups 36−40 years and 21−25 years respectively ($P > 0.05$). There was a statistically significant association between the presence of both viruses and women in polygamy and those who have undergone blood transfusion and surgery ($P < 0.05$).
Conclusions: The results obtained in this study showed a considerably higher sero-prevalence rate of HIV and HBsAg amongst the pregnant women. Hepatitis B virus infection is a dynamic disease and co-infection with HIV impacts directly on the outcome of HBV infection, considerably complicating its natural history, diagnosis, and management, Therefore screening for HIV and HBsAg co-infection during pregnancy is essential to improve ante-natal care and inform clinical management.

P1030
Different prognostic value of cell-associated total HIV-1 DNA and integrated HIV-1 DNA for virological outcome in ART naïve and treated HIV-1 chronically infected patients

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Purpose/Objective: It has been demonstrated that total HIV-1 DNA levels associated to peripheral blood mononuclear cells (HIV-1 DNA_T) has prognostic value for virological outcome in HIV-1 infected patients. HIV-1 DNA_T includes both integrated and unintegrated forms of HIV-1 DNA. During natural infection the majority of HIV-1 DNA exists in an unintegrated state, compared with treated patients where the majority DNA is integrated. We evaluated whether the integrated forms of HIV-1 DNA (HIV-1 DNA_I) could provide a different prognostic value compared to HIV-1 DNA_T for virological outcome in ART naïve chronically HIV-1^+ individuals.
Materials and methods: In 107 ART naïve HIV-1^+ patients who have been previously analyzed for HIV-1 DNA_T we have retrospectively measured integrated DNA_I using real time PCR and relative quantification by $\Delta\Delta$Ct. Patients had participated in a multicenter, randomised, double-blinded, placebo-controlled phase II clinical trial of ART combined with an HIV-1 immunogen (STIR-2102). We analyzed the prognostic value of HIV-1 DNA_T and HIV-1 DNA_I as categorical variables using bivariate Cox regression models and stratifying by the medians at baseline PRE-ART and POST-ART (6 weeks after initiation ART). In STIR-2102 trial the endpoint was defined as time to the first increase of HIV-1 RNA > 5000 copies/ml.
Results: HR for virological failure in PRE-ART patients, introducing in the Cox model HIV-1 DNA_T and HIV-1 DNA_I were: HR 1.69 (95% CI, 1.02−2.79, $P = 0.04$) and HR 1.83 (95% CI, 1.11−3.03, $P = 0.019$), respectively. In POST-ART HR for HIV-1 DNA_T and HIV-1 DNA_Iwere: HR 2.36 (95% CI, 1.39−4.02, $P = 0.001$) and HR 1.68 (95% CI, 1.01−2.80, $P = 0.046$), respectively.
Conclusions: Both HIV-1 DNA_T and HIV-1 DNA_Ishowed different independent prognostic value. In ART naïve patients integrated HIV-1 $DNA_I$was the strongest variable associated with virological failure independently of HIV-1 DNA_T. In treated patients, HIV-1 DNA_T showed the strongest prognostic value for virological outcome, probably reflecting residual replication.

P1031
Dissecting HIV-1 clade C − specific antibody responses

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Purpose/Objective: Out of the different HIV strains, HIV-1 clade C is the one which is expanding more rapidly, above all in the southern states of Africa. Though, little is known about the immune response elicited by this strain in HIV infected individuals. In order to dissect the clade C HIV-1-specific antibody response in HIV-infected individuals we produced recombinant proteins and synthetic peptides.
Materials and methods: For a precise mapping of epitopes lying on the HIV envelope proteins (gp120 and gp41) overlapping peptides covering the entire amino acid sequence of the two proteins of a South African HIV-1 clade C strain were synthesized by solid phase synthesis. In order to indentify epitopes on non-surface antigens the HIV-1 clade C structural, functional and accessory proteins have been expressed in *Escherichia coli*. In preliminary experiments 85 sera from Zimbabwe were tested for HIV-specific IgG reactivity to the produced antigens in a dot blot immunoassay.
Results: The envelope epitope mapping allowed identifying immunogenic regions of gp120 and gp41. Interestingly HIV-specific IgG antibodies have been found to be elicited by both the domains which are conserved among HIV-1 strains as well as by variable domains (i.e. C1, V3 and V4 regions). On the other hand, we detected strong IgG reactivity also to proteins and peptides which have not been reported to be exposed on the surface of the virion (i.e. gp41 C-terminal region, matrix protein, Vif).
Conclusions: This suggests that also proteins contained within viral particles are exposed to the immune system in an immunogenic form.

P1032
Effectiveness of herbal remedy in 6 HIV patients in Nigeria

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Purpose/Objective: The uncurable nature of HIV infection compelled many people living with the virus to seek alternative therapy. This pilot study determined effectiveness (clinical and laboratory responses) of an herbal remedy (A-Zam) used by patients seeking herbal remedy for HIV infection in Nigeria.
Materials and methods: Six patients taking herbal concoction as alternative therapy for HIV infection were recruited into the study and monitored for 4 months. All the (6) patients were confirmed for presence of HIV infection using Western blot technique in the nearest teaching hospital before commencing preliminary clinical and laboratory examinations using WHO and CDC criteria. The patients were contacted daily and visited regularly after commencement of herbal medications to assess side effects, drug toxicity, compliance and effectiveness.
Results: The symptoms and signs associated with HIV infection disappeared within 20 days of commencement of herbal therapy. The body weight increased from average 52.8kg to 62.7kg (9.9 ± 3.2), viral (HIV-RNA) load decreased from average 42 300 copies/ml to undetectable level (≤50 copies/ml) and CD4 T cell count increased

from average 226–680 $mm^3/\mu l$ (454 ± 106 $mm^3/\mu l$) at 4-month post therapy.
Conclusions: This pilot study concluded that the herbal remedy (A-Zam) is effective in HIV infection based on dramatic improvement in both clinical features and laboratory results of HIV infection. However, a longer period is suggested to ensure that the observed improvement is sustained. Also, a large population study is needed to confirm our observation in this cohort of people

P1033
Evaluation of the presence of the compound KIR:HLA-C genotypes in the virologic outcome of individuals with HIV-1 chronic infection

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Purpose/Objective: An effect of certain HLA-C genotypes in combination with KIR2DL3 has been implicated with HCV clearance previously. However, to date neither individual HLA-C alleles nor combinations or HLA-C groups with KIR have shown a protective effect against HIV-1 infection. The objective of this work was to study whether the presence of different compound KIR:HLA-C genotypes (KIR2DL1 and its ligand the HLA-C2 allotype; KIR2DS1:HLA-C2; KIR2DL2:HLA-C1; KIR2DL3:HLA-C1; and KIR2DS2:HLA-C1) could influence the virological outcome of chronically HIV-1 infected individuals initiating antiviral-therapy.
Materials and methods: Of the 187 patients with asymptomatic HIV-1 chronic infection who had participated in a multicenter, randomised, double-blinded, placebo-controlled phase II clinical trial of ART combined with an HIV-1 immunogen (STIR-2102), were retrospectively genotyped for HLA-C allotypes and KIR genes using sequence-specific primer PCR (Olerup SSP KIR Genotyping kit). Kaplan–Meier curves and Cox proportional-hazard models were used for survival analysis.
Results: Kaplan–Meier analyses, stratifying by the different genotypes showed that HLA-C1 allotype and KIR2DL3 were strongly associated with virological failure (χ^2 value for the Log-Rank test was 8.46; $P = 0.004$): The mean time to virological failure (first increase of HIV-1 RNA above 5000 copies/ml) was 31.60 months (95% CI, 29.29–33.92) for the group KIR2DL3:HLA-C1 and 20.74 months (95% CI, 19.07–27.06M) for the group of patients lacking the KIR2DL3:HLA-C1 genotype. In a Cox regression model, the HR for virological failure was significantly higher for individuals without the compound KIR2DL3:HLA-C1 genotype [HR, 1.90 (95% CI, 1.20–2.97, $P = 0.006$)].
Conclusions: The compound KIR2DL3:HLA-C1 genotype has been associated to a protective effect in a cohort of chronically HIV-1 infected individuals initiating antiviral-therapy.

P1035
High numbers of M-DC8+ monocytes and TNFa+ cells within the marginal zone of spleens from HIV-1 infected patients

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Purpose/Objective: We found recently that circulating M-DC8+ monocyte numbers were significantly higher in viremic, HIV-1 infected patients than in non-viremic patients or in healthy controls. *In vitro*, peripheral blood mononuclear cells from viremic patients produced more TNFα in response to LPS than those from non-viremic patients or from controls, and M-DC8+ monocytes were mostly responsible for this overproduction. We quantified and localized these monocytes in spleens from HIV-1 infected patients with idiopathic thrombopenic purpura (ITP).
Materials and methods: Samples were obtained after therapeutic splenectomy from HIV-infected or uninfected patients. Numbers of M-DC8+ monocytes were evaluated by 11-color flow cytometry in spleen mononuclear cells from 10 patients (six HIV-infected, four uninfected). Localization and quantification of M-DC8+ and TNFα+ cells were performed by immunohistofluorescence on spleen cryosections from 17 patients (eight uninfected, nine HIV-infected including four untreated by antiretrovirals).
Results: Spleens from HIV-infected patients displayed significantly higher numbers of M-DC8+ monocytes than those from uninfected patients. M-DC8+ cells were localized in the red pulps from all patients, but were present within the marginal zone only in HIV-infected, untreated patients. Numbers of TNFα+ cells were also significantly higher in HIV-infected patients than in controls, irrespective of treatment. Some were found within the marginal zone, whereas in uninfected patients, they localized strictly in the red pulp. TNFα did not colocalize with M-DC8+ cells, but with other cell types currently being characterized.
Conclusions: High numbers of M-DC8+ monocytes were not only found in the blood from HIV-infected patients, but also in the spleen. In untreated patients, these cells localized abnormally within the marginal zone. TNFαexpression was higher in spleens from HIV-infected patients than in uninfected patients, and was found within the marginal zone, but did not colocalize with MDC8 labeling. We will test further the kinetics of proinflammatory monocyte homing in a simian model over different stages of SIV infection.

P1036
Highly Active Antiretroviral Therapy (HAART) can restore the decrease of CD20+ T cell numbers in HIV-1 patients

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Purpose/Objective: CD38 expression on $CD8^+$ T cells is a valuable prognostic marker for disease progression in HIV infection. Like CD38, CD20 is functionally involved in calcium mobilization and coexpressed by a subpopulation of human T cells. Here we wanted to elucidate if $CD20^+$ T cells are affected by HIV-1 infection and may have a prognostic value for the course of disease.

Materials and methods: Numbers of $CD20^+$ T cells were determined in healthy controls, untreated and HAART-treated HIV-1 patients. Coexpression patterns of CD4, CD8, and CD38 as well as IFN-γ production were analysed in $CD3^+$ $CD20^+$ and $CD3^+$ $CD20^-$ T cells by multi color flow cytometry.
Results: We found a significant decrease of $CD20^+$ T cell numbers in untreated HIV-1 patients (1.4%) as compared to healthy controls (2.5%) which recovered under HAART (1.9%). Particularly, the $CD8^+$ T cell compartment was affected revealing significant differences between healthy controls (3.4%) and both treated (1.7%) and untreated (1.1%) patients. CD38 was expressed on a few $CD20^+$ T cells but preferentially on $CD20^-$ cells in all three groups.

IFN-γ production was measured upon cell activation using PMA alone or in combination with ionomycin in order to assess functional capacities of the cells. PMA alone was much more effective in $CD20^+$ cells regardless of CD38 coexpression, indicating a supportive role of CD20 but not CD38 in T cell activation.
Conclusions: Here we present data showing that $CD3^+$ $CD20^+$ T cells are decreased in untreated HIV-1 patients and normal numbers are restored under HAART. Expression of CD20 and CD38 is independently regulated on T cells and it is not clear by now if CD20 expression is of prognostic value. Contrary to CD38, CD20 can substitute ionophores for Ca^{2+} flux in early T cell activation and also strongly amplifies cell stimulation in the presence of Ca^{2+} ionophores, indicating that CD20 contributes to T cell activation.

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P0137
HIV impairs avidity maturation to EPI vaccines in children

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Purpose/Objective: HIV-infected adults and children have reduced quantities of antibodies and memory B cells to non-HIV antigens. On the other hand, avidity of antibodies to vaccine antigens is not affected by HIV in adults, probably because they have already experienced avidity maturation to most routine vaccine antigens prior to infection. Little is known regarding avidity maturation in children infected with HIV. Here, we analysed the avidity of antibodies against tetanus toxoid and diphtheria toxoid in children who acquired HIV vertically. We also measured the quantities of circulating antibodies and frequencies of memory B cells to vaccine antigens.
Materials and methods: Memory B-cell frequencies were determined using cultured B-cell ELISpot. ELISA was used to determine the antibody levels while a modified ELISA was used to determine the antibody avidities. In the modified ELISA, Guanidine Hydrochloride was used to elute the antibodies after incubating the plasma (sample) with the antigen-coated plates. The ratio of the remaining antibody levels in the eluted wells to that in the control (PBS eluted) wells was taken as the avidity index.

The children were then classified based on their viral loads and compared with community controls.
Results: HIV-infected children, regardless of level of viremia, had significantly lower avidity indices when compared with the community controls. As previously reported, they also had lower titres of circulating antibodies as well as lower frequencies of antigen specific memory B cells.
Conclusions: HIV affects not only the magnitude but also the quality of antibody response to vaccine antigens in vertically infected children.

P0138
HIV triggers interleukin 21-mediated induction of granzyme B-secreting B cells with antiviral properties

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Purpose/Objective: Certain lymphocyte subsets including plasmacytoid dendritic cells and regulatory T cells can secrete granzyme B (GrB), thereby suppressing T cell expansion. Recently, we found that B cells can also produce GrB in response to interleukin (IL-) 21. Since HIV has been shown to be associated with elevated serum IL-21 levels, we hypothesized that GrB-expressing B cells may be induced during HIV infection.
Materials and methods: Cell culture, HIV infection of cells in cell culture, FACS, laser-scanning confocal microscopy, Wetern Blotting.
Results: Here, we demonstrate for the first time, that infection of $CD4^+$ T cells with HIV 1 (NL4-3), but not mock infection, induces strong expression of IL-21. We further demonstrate that such T cells induce GrB in co-cultured B cells in an IL-21-dependent fashion. In support of these data, serum levels of both IL-21 and GrB are significantly higher in HIV-infected patients before HAART as compared to healthy controls. Up to 60% of B cells ($36.2 \pm 12.9\%$) from patients infected with HIV, but not normal B cells, express GrB. Importantly, co-culture of HIV-infected $CD4^+$ T cells with GrB^+ B cells resulted in GrB transfer, and strongly suppressed both, proliferation of T cells and virus replication as indicated by significantly reduced p24 levels in the supernatants. The observed effects were enhanced by IL-21, and reduced by GrB inhibition.
Conclusions: In summary, we demonstrate that HIV infection induces IL-21 in $CD4^+$ T cells, thereby indirectly triggering the development of GrB-secreting B cells with antiretroviral properties. GrB-secreting B cells may play a so far unappreciated role in decelerating HIV expansion, particularly in the early phase of infection. On the other hand, induction of GrB in B cells may interfere with their terminal differentiation into plasma cells, which may explain the lack of an efficient anti-HIV humoral immune response in HIV-infected patients.

P1041
Immunogenicity of a universal HIV-1 vaccine vectored by DNA, MVA and ChAdV-63 in a phase I/IIa clinical trial

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Purpose/Objective: The develop a safe and effective vaccine against HIV-1/AIDS.
Materials and methods: The major challenge that both antibody and T cell-eliciting vaccines against HIV-1 face is the extreme variability of the HIV-1 genome: a successful vaccine has to effectively target diverse HIV-1 strains circulating in the population and then must deal with ongoing virus escape in infected individuals. To address these issues, we assembled vaccine immunogen HIVconsv from the functionally most conserved regions (not epitopes) of the HIV-1 proteome with the underlying working hypothesis that early focus of vaccine-elicited immune responses on these regions will lead to a better recognition

and control of transmitting viruses. A gene coding for the HIVconsv immunogen was inserted into plasmid DNA (D), modified vaccinia virus Ankara (MVA; M) and non-replicating adenovirus of a chimpanzee origin ChAdV-63 (C). Currently, combined heterologous prime-boost regimens of these vaccines, namely CM, DDDCM and DDDMC, are being evaluated in a phase I/IIa trial HIV-CORE002 in healthy HIV-1/2-negative volunteers in Oxford.
Results: Preliminary data indicate that the vaccines are very well tolerated and show high immunogenicity. Following the CM regimen, vaccine-induced T cell frequencies reached a median of 5150 (range 1475−16495) SFU/10^6 PMBC *ex vivo* specific for multiple, conserved, therefore in natural infection mostly subdominant HIV-1 epitopes (in contrast to 136 and 686 SFU/10^6 PMBC in the STEP study). Similar immunogenicity was observed for the DDDCM regimen. Results from DDDMC, epitope mapping, and phenotypic and functional characterization of these responses will be presented.
Conclusions: Thus so far, the unique HIVconcv immunogen and unique vector delivery have induced T cell responses superior to other HIV-1 vaccine candidates tested in humans to date, HIVconsv is the first of the second-generation immunogens addressing the HIV-1 diversity that reached clinic and ChAdV-63 is the first adenovirus of chimp origin delivering an HIV-1-derived immunogen that reached clinic.

P1042
Immunomodulation of antigen-specific T cells by HIV gp41 transmembrane envelope protein

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Purpose/Objective: Modulation of T-cell responses by human immunodeficiency virus (HIV) occurs via distinct mechanisms, one of which involves down-regulation of T cells already at the stage of virus-cell fusion. The membrane-bound T-cell receptor (TCR) complex is located in the vicinity of the HIV fusion site. Thus, it is conceivable that hydrophobic portions of the gp41 protein of the viral envelope that contributes to membrane fusion may interact with membrane-embedded portions of the TCR and modulate T-cell responsiveness.
Materials and methods: To address this hypothesis, we investigate the ability of HIV gp41 derived peptides to modulate T-cell proliferation *in-vitro* by examining their effect on T cells, which were stimulated at different steps of the TCR complex signal transduction. Furthermore, imaging, biochemical and biophysical approaches are implemented to identify the target protein of the HIV peptides. *In-vivo* studies are carried out in mice models of autoimmune disease.
Results: We demonstrate inhibition of antigen-specific T-cell proliferation by peptides derived from membranotropic regions of HIV gp41. We will address the mode of action of the peptides on the transmembrane domains of the TCR complex. Furthermore, administering the peptides to an animal model of autoimmune disease mediated by pathogenic T-cells significantly reduces the severity of the disease. This seems to be associated with down-regulation of Th1 pro-inflammatory cytokines.
Conclusions: Thus, our data imply that T-cell inactivation during HIV-cell fusion lie in part in hydrophobic portions of gp41. Disassociated from HIV, however, these HIV peptides may act as novel reagent for down-regulating undesirable immune responses.

P1044
Inhibition of HIV-replication by cell-membrane crossing oligomers (CMCOs)

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Purpose/Objective: Although rapidly becoming a valuable tool for gene silencing, regulation or editing *in vitro*, the direct transfer of siRNAs into cells is still an unsolved problem for *in vivo* applications.
Materials and methods: For the first time, we show that specific modifications of antisense oligomers allow autonomous passage into cell lines and primary cells without further adjuvant or coupling to a cell-penetrating peptide. For this reason, we termed the specifically modified oligonucleotides '*cell-membrane crossing oligomers*' (*CMCOs*).
Results: CMCOs targeted to various conserved regions of HIV-1 were tested and compared to non-targeting CMCOs. Analyses of non-infected and infected cells incubated with labeled CMCOs revealed that the compounds were enriched in infected cells and some of the tested CMCOs exhibited a potent antiviral effect. Finally, the CMCOs did not exert any cytotoxicity and did not inhibit proliferation of the cells.
Conclusions: *In vitro*, our CMCOs are promising candidates as biologically active anti-HIV reagents for future *in vivo* applications.

P1045
Interferon alpha induced acceleration of thymic production during acute SIV infection

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Purpose/Objective: The processes involved in the inhibition of thymic function usually observed during primary HIV-infection are poorly understood. In humans, the acute phase of HIV-infection, that certainly drives pathogenesis, remains barely studied. The aim of this study was to define the mechanisms impacting thymopoiesis during the acute phase of SIVmac251-infection in rhesus macaques.
Materials and methods: Blood samples were taken every other day for 2 weeks in 15 SIV-infected macaques. T-cell subsets were followed by flow cytometry. Plasma interferon alpha (IFNa) levels were measured by ELISA. Thymic function was evaluated by qPCR quantification of T-cell Receptor Excision Circles (TRECs) and estimation of intrathymic precursor T-cell proliferation (sj/§TREC ratio). Eight animals were autopsied at day 3, 7, 10 and 14 post-infection. Thymic expression of IFNa was evaluated by qRT-PCR and the proportion of the 12 IFNa subtypes estimated by heteroduplex tracking assay. The expression of chemokines involved in thymopoiesis was studied by qRT-PCR on thymus samples. Finally, the effect of IFNa subtypes on thymocyte differentiation was tested on simian DN cells cultured on OP9-hDL1 cells.
Results: Combined analysis of the evolution of recent thymic emigrants (CD31+ naïve CD4+ T-cells) in blood and TRECs pointed to the fact that, by day 7 of infection, the RTE compartment mostly contains newly exported cells with a reduced proliferation history. This change in thymocyte behavior coincided with both increased plasma IFNa levels and production of IFNa 1, 2, 3, 5 and 7 in the thymus. In culture, 6 IFNa subtypes, including IFNa3 and 7, induced strong inhibition of thymocyte proliferation, as demonstrated by lower cell counts, DP frequencies and sj/bTREC ratio at day 14. Finally, in the SIV-infected thymuses CXCL12 transcription was significantly increased while CCL19 and CCL25 expression was reduced.

Conclusions: Altogether, our data demonstrate that, during acute SIV-infection, modified chemokine expression patterns in the thymus and inhibition of thymocyte proliferation by locally produced IFNa subtypes lead to an acceleration of thymocyte differentiation thus to massive export of newly maturated T-cells. In the long term, such modification of thymopoiesis may lead to the observed reduction of naïve T-cell counts and diversity.

P1046

M-DC8+ monocyte expansion and TNFa overproduction in response to LPS during HIV-1 infection

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Purpose/Objective: HIV infects activated CD4+ T cells and induces their depletion. Progressive HIV infection is fueled by chronic immune hyperactivation, mediated by inflammatory cytokines like TNFα, and related to intestinal epithelial damage and microbial LPS translocation into the circulation.
Materials and methods: Using 11-color flow cytometry and cell culture after sorting, we investigated the numbers and TNFα production of fully defined myeloid populations during HIV-1 infection.
Results: In 15 viremic patients, compared to 8 virologically suppressed patients or to 13 controls, circulating CD141 (BDCA-3)$^+$ and CD1c (BDCA-1)$^+$ dendritic cell counts were reduced. Conversely, non-classical CD14^{dim}CD16$^+$ monocyte counts were increased, particularly those expressing M-DC8. These M-DC8$^+$ monocytes were mostly responsible for the LPS-induced TNFα overproduction found in viremic patients. *In vitro*, CD16$^+$M-DC8$^+$ monocytes differenciated from classical, CD16$^-$ M-DC8$^-$ monocytes using M-CSF and GM-CSF, which is increased in viremic patient's plasma.
Conclusions: This M-DC8$^+$ population, which is involved in the pathogenesis of chronic inflammatory diseases, might thus be considered as a major actor in the vicious circle of immune hyperactivation fueling HIV infection progression.

P1047

mCMV infection can be beneficial or harmful in retrovirus infection dependent on the order and time-point of infections

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Purpose/Objective: Suppression of the immune system e.g. transplantation or HIV infection can result in reactivation of cytomegalovirus (CMV). In general, CMV reactivation can be life-threatening and therefore it should be avoided especially in transplantation patients. However, recently it was shown that CMV reactivation early after bone marrow transplantation in human adult myeloid leukemia can also be beneficial by reducing tumor relapse, indicating that CMV infection can be beneficial for the host. Different to CMV reactivation studies in AIDS patients, only limited studies are done to investigate if a primary or persistent CMV might be beneficial or harmful in HIV patients.
Materials and methods: In this study we used the two well established infection models in mice, the Friend leukemia Virus (FV), as a model for retroviral infections and the murine cytomegalovirus (mCMV), as a model for human cytomegalovirus infections.
Results: A primary mCMV infection resulted in enhanced FV replication in mice persistently infected with FV and enhanced numbers of functional FV-specific CD8 T cells. However these cells become dysfunctional suggesting that expanded Treg cells might dampen the 'newly' generated FV-specific T cell response and inhibit the clearance of persistent FV infection.

In contrast to these findings, we found that persistent mCMV limited a primary FV infection. Significantly reduced FV titers were found during a primary FV infection in persistently mCMV infected mice compared to primary FV infected naïve mice. The reduced FV titers were mediated by an augmented FV-specific CD8 T cell response. In persistent mCMV infection we found that FV-specific CD8 T cells had a significant earlier and stronger *in vivo* killing capacity during primary FV infection compared to only primary FV infected mice.
Conclusions: In conclusion, our data showed that a primary mCMV infection is harmful in persistently retrovirally infected mouse, but a persistent mCMV infection is beneficial by reducing viral titers of a primary retrovirus infection. Our data demonstrated that the mCMV mediated effect onto a retroviral infection is dependent on the sequence order and the time-point of mCMV infection.

P1048

Modifications of the redox intracellular environment during the Human Immunodeficiency Virus type I (HIV-1) infection associated with the Src kinase activation

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Purpose/Objective: Human Immunodeficiency Virus (HIV) and other structurally simple retroviruses developed, as a defense mechanism, numerous replication strategies to escape from apoptotic mechanism triggered by host cells. HIV maintains a persistent infection by stimulating intracellular (host) production of cytokines and of reactive oxygen and nitrogen species (ROS and RNS) (1). Levels of ROS and RNS and of their antioxidant counterparts determine the redox environment. Alterations on the redox environment are sensed by raising levels of the antioxidant enzyme Cu/Zn and Mn Superoxide dismutase (SOD). Such alterations are potentially associated with the regulation ofHIV-replication signaling pathways (2).
Materials and methods: In the present study we used isolated human CD4 T lymphocytes submitted to a protocol of *in vitro* infection with purified HIV viral particles. Levels of the antioxidant enzymes, Cu/Zn and Mn-SOD, Glutathione peroxidase (GPx), Glutathione reductase (GR), and of the tri-peptide Glutathione (GSH) were measured in infected and non-infected CD4 T lymphocytes.
Results: There was an increase on the activities of the antioxidant enzymes followed by a decrease on intracellular GSH levels. In addition we followed the temporal pattern of activation of Src kinase. Src is a cytoplasmic protein tyrosine kinase which has been shown to play an important role in HIV-1 replication (3).
Conclusions: Our findings suggest a possible relationship between the alterations in intracellular redox environment and the activation pattern of Src kinase in HIV-infected human CD4 T lymphocytes. These results also emphasize the importance of the intracellular redox environment in regulating the Src-mediated signaling pathway associated with HIV infection.

P1050

Natural T regs recently emigrated from the thymus; consist of two subsets, differently impaired during HIV infection

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Purpose/Objective: The hallmark of HIV infection is the sustained deterioration of the CD4 T cell compartment accompanied by the exhausted thymic function. The role of different Treg populations in the course of HIV infection remains imprecisely determined with nTregs being less characterized than iTregs due to the lack of a clearly defined specific markers. However, it can be expected that similarly to non-Treg naïve T-cells, HIV infection impacts on *de novo* production of nTregs.

Materials and methods: Of the 26 healthy control (HC) individuals and 16 age/sex matched HIV-1+ subjects, ART-treated or therapy-free, were included. PBMCs were simultaneously stained for extra and intracellular markers. The determination of nTregs-RTE cells was done by multi-colors flowcytometry using combinations of anti-CD3, -CD4, -CD45RA, -CD31, -CCR7, -CD25 and -CD127. TREC quantification was performed by real-time PCR on FACS-sorted lymphocytes and expressed as TRECs/10^5 cells. Statistical analysis was performed using GraphPad software.

Results: In the compartment of naïve CD4+T cells ($CD45RA^+$ CCR7+) we identified two subsets of recent thymic emigrants (RTE) nTreg cells (CD31+FOXP3+ naïve CD4+ T-cells) differentially expressing CD25. These 2 subsets contained high concentrations of TRECs, confirming thymic proximity. In contrast, no difference in TREC levels was observed between CD25+ and CD25- nTregs-RTE. In HCs CD25+ nTregs-RTE were twice as numerous as CD25- nTregs-RTE ($P = 0.027$). In patients' groups an increase of RTE nTregs was observed, mostly as a consequence of CD25- nTregs-RTE expansion ($P < 0.02$). In ART-treated patients, they essentially consisted of CD127 cells ($P < 0.05$). This was not the case in the group of untreated patients ($P > 0.05$).

Conclusions: Our results show that nTregs recently emigrated from the thymus consist of two subsets differentially expressing CD25. These subsets are differently impaired during HIV-infection. Further studies are needed to clarify their respective functions and behaviors in the course of HIV infection.

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P1051

Selection of sero-conversion predictive marker by serological analysis of HIV-diagnosed cases in Korea

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Purpose/Objective: Even though the latest serological or molecular methods are used to diagnose HIV infection, it is still difficult to determine the HIV status of indeterminate cases. In this study, we identified HIV status of Korean sero-converter in HIV low prevalence area and chose the most relevant markers to predict the status of sero-conversion by analyzing serological characteristics of HIV sero-converters.

Materials and methods: We analyzed the results of the serological tests for HIV diagnosis in Korea from 2009 to 2011. The results of HIV diagnosis were classified as positive, indeterminate, and negative. Especially, we evaluated the predictive value of each antibody profile with presence of p24 antigen based on serological data of sero-converters who were assessed as indeterminate at first, retrospectively.

Results: From 2009 to 2011, 2429 people were diagnosed as HIV infected individuals in Korea, and 199 of those cases (8.2%) were determined as sero-converters. Among 199 sero-converters, up to 72.2% (60/83) showed Ab-/Ag+ in screening test were sero-converted. In the case of sero-conversion, the use of antigen detection and western blot analysis, such as the group of Ag+/gp160 (OR; 88.3) orAg+/p24 (OR; 17.9), had higher positive predictive value than the Ag+ group (OR; 9.3) of indeterminate cases.

Conclusions: According to our study, the HIV antigen detection and appearance of western blot profile to gp160 protein could be used as a high predictive diagnostic marker to determine the HIV status of indeterminate at first. These results would be helpful to increase the specificity of the HIV positive criteria in HIV low prevalence region without delayed interpretation of HIV status.

P1052

Polymorphisms in the HCP5 and HLA-C genes associate with time to undetectable plasma HIV RNA during highly active antiretroviral therapy (HAART)

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Purpose/Objective: Single nucleotide polymorphisms (SNPs) in the HLA complex P5 gene (*HCP5*), *HLA-C,* and near the zinc ribbon domain containing 1 gene (*ZNRD1)* have been shown to influence viral load (VL) set point in HIV-infected individuals with known time of seroconversion. We aimed to determine the influence of the *HCP5* rs2395029, the *HLA-C* rs9264942, and the *ZNRD1* rs3869068 on mean VL before HAART, on time to first VL < 51 copies/ml, and on CD4 T-cell recovery during HAART.

Materials and methods: The Danish HIV Cohort Study is a prospective, nationwide, population-based study of all HIV-infected individuals treated in Danish HIV clinics since 1 January 1995. From this cohort we genotyped 1382 Caucasian individuals for the rs2395029 (A>C), rs9264942 (T>C), and rs3869068 (C>T).

We calculated the mean viral load before HAART for each individual with a CD4 T-cell count >200 cells/μl and calculated the CD4 T-cell recovery as the weighted average of the last CD4 T-cell count before 1 year with HAART and the first CD4 T-cell count after 1 year with HAART minus the last CD4 T-cell count before HAART. General linear models were applied to evaluate the effect of SNPs on mean VL before HAART and on CD4 cell recovery during HAART. Cox proportional hazard regression analysis was applied to assess the association with time to first VL < 51 copies/ml. All models were assuming additive or dominant genetic effects and were adjusted for

sex, age, calendar period, and start of HAART (where appropriate). VL was log-transformed before analysis.
Results: The C-allele of rs2395029 was associated with lower mean VL before HAART [(mean ± SD), CC/CT: 4.1 ± 0.95 versus TT: 4.6 ± 0.83, $P = 0.0003$]. We found no significant associations with mean VL before HAART for the rs9264942 and rs3869068. The C-alleles of rs2395029 and rs9264942 were associated with a shorter time to VL < 51 copies/ml [HR (95% confidence interval) = 1.36 (1.10−1.69), $P = 0.005$; HR = 1.17 (1.07−1.28), $P = 0.0004$, respectively]. None of the SNPs predicted CD4 T-cell recovery during HAART.
Conclusions: The C-allele of rs2395029 associates with mean VL before HAART and the C-alleles of rs2395029 and rs9264942 associate with time to first VL < 51 copies/ml during HAART further emphasizing the impact of these SNPs on viral replication.

P1053
Precursor frequency of HLA-B27-restricted HIV KK10-specific CD8+ T-cells is not related to their immunodominance

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Purpose/Objective: It has previously been demonstrated that HLA-B27 allele is associated with the control of HIV replication and that HIV specific CD8+ T cell responses restricted by this HLA molecule are immunodominant and present superior functional attributes. Studies performed in mice have shown that the frequency of naïve precursors impacts on the quantitative and qualitative attributes of CD8+ T cells during immune responses. We therefore hypothesized that advantageous properties of HLA-B27 restricted HIV specific CD8+ T cells may be linked to the initial frequency of naïve precursors.

The objective of this study was to calculate the frequency and priming capacity of naïve CD8 T cells specific for HIV epitopes restricted by the protective (HLA-B27, KK10) and the non-protective HLA alleles (HLA-A2, S9L or HLA-B7 RL9).
Materials and methods: Our data was generated using blood samples derived from healthy HIV-seronegative subjects. HIV reactive $CD8^+$ T-cell precursors were enriched from 10^8 peripheral blood mononuclear cells (PBMCs) using cognate peptide-HLA class I tetramers and magnetic beads, and their frequency was calculated. We also expanded naïve $CD8^+$ T-cells by performing *ex vivo* priming with peptide-pulsed autologous dendritic cells, which were differentiated using GM-CSF and IL-4 and matured with a cytokine cocktail for 20 days in culture. For comparison, we performed the same assays for HLA-A2 restricted Melan-A (EV10) reactive $CD8^+$ T-cells, known for their high frequency of naïve precursors.
Results: The frequency of Melan-A reactive $CD8^+$ T-cell precursors was >100 cells per million $CD8^+$ T-lymphocytes, In contrast, the frequency of KK10 reactive precursors was approximately 1 cell per million $CD8^+$ T-lymphocytes and was not different from those measured for other HIV specificities. These findings were confirmed using the *in vitro*expansion assay.
Conclusions: Our results suggest that the frequency of naïve precursors *per se,* cannot explain the acquisition of superior functional attributes or the immundominance of KK10/HLA-B27-specific $CD8^+$ T-cell populations in individuals infected with HIV-1.

P1054
Rapid broadening of the CTL responses retards the evolution of immune escapes of HIV

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Purpose/Objective: During the first months of infection with HIV the virus typically evolves immune escape mutations. These escape mutations are found in epitopes from the virus and reduce the selection pressure of CD8+ T cell responses specific for these epitopes. Recent data suggest that most of these immune escape mutations evolve early and rapidly. By using a mathematical model, we try to understand why the evolution of immune escape slows down over the time of infection, and how immune escapes depend on the breadth of the immune response to the virus.
Materials and methods: We used a conventional mathematical model with the main distinguishing feature that several immune responses can together control the virus at steady state (e.g. at the viral set-point). Individual CTL responses appear at different time points during infection.
Results: We find that most escapes occur early, when the diversity of the immune response is still small. A generic feature of the model is that the contribution of each immune response at steady state declines when the number of immune responses increases. Escaping one immune response provides little advantage when the breadth of immune responses is high. The impact of the breadth of the CTL response is even stronger if some of the viral epitopes are difficult to escape and/or the virus has a poor replicative capacity.
Conclusions: The contribution of a single CTL clone decreases when the breadth of the CTL response increases. Escapes therefore tend to happen during early infection because the breadth of the CTL response is still small.

P1055
Single amino-acid change in a highly conserved motif of the gp41 elicits viral neutralization and protects against CD_4 depletion

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Purpose/Objective: The hallmark of most successful vaccine is the ability to induce cross-reactive neutralizing antibodies (Nabs). For HIV-1, only a handful of broadly Nabs (bNAbs) have thus far been identified. Over the ensuing years, we have shown that a highly specific and conserved motif of the gp41, called 3S, induces expression of NKp44L, the cellular ligand for an activating NK receptor on CD4 T cells render them more sensitive to autologous NK lysis. The aim of this study was to explore the possibility that substitutions inside this 3S motif could permit the development of bNAbs while preserving the capacity to block CD4 depletion.
Materials and methods: To further characterized the 3S peptide, point mutations were introduced inside the 3S motif of the gp41. We tested such effect on the capacities to infect cells and the ability of the corresponding peptides to induce production of Ab that elicit viral neutralization and/or inhibit NKp44L expression on CD4T cells and NK cell function.
Results: In this study, alanine-scanning allowed us to identify specific positions in the 3S motif that inhibit HIV entry and expose it to a broad spectrum of neutralizing antibodies. Importantly, for the first time, we show that specific amino-acid substitutions within a highly linear motif of the gp41 elicit strong neutralization capacity with

impressive magnitude, breadth and ability to durability over cross clade viruses. Furthermore, our data also show that this acquisition of neutralization capacities preserves the unique ability of anti-3S Ab to inhibit NKp44L expression on CD4+T cells and their sensitivity to NK lysis.

Conclusions: Our finding suggest that specific substations into the 3S based immunogens may lead to the generation of bNAbs directed against the 3S conserved motif of gp41 and will provide foundation for a vaccine design based on 'bi-functionals' Abs allowing both viral neutralization and CD4 protection.

P1056

Study on the Functional role of Immunoglobulin E as surrogate marker for HIV/AIDS infection

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Purpose/Objective: IgE class of antibodies has been found in mammals and plays an important role in allergic and hypersensitivity reactions. Certain viral infections are known to produce specific IgE antibodies, to the extent that significant changes in the level of total serum IgE may occur. Study attempts to associate the Level of Ig E in HIV progression.

Materials and methods: The study involves fifty HIV seropositive patients attending Anti-Retroviral Therapy Centre, Department of Sexually Transmitted Disease, Rajaji Government Hospital, and Madurai, India subjected for the present study. The individual involves 27 HIV/AIDS Male patients, 23 HIV/AIDS Female patients. The control sample comprises 15 HIV sero negatives. The samples were collected at the informed consent of the patients. Serum sample were collected and IgE was quantified using MAGIWELL IgE quantitative solid phase Enzyme linked Immunosorbent assay (ELISA).

Results: The study documents highest percentage of deviation from the control observed in Male HIV seropositives (43.7%) and age-wise influence documents highest percentage of deviation in the age group 15–29 years (56%).

Conclusions: Serum IgE level in the present study found to be elevated from the normal range documents the existence of imbalance between Th 1 and Th 2 and associated with T-cell dysfunction and a hypergammaglobulinemia. The present results suggest that elevation of circulating IgE levels may be due, at least in part, to specific IgE directed to the HIV virus rather than as a result of a nonspecific phenomenon. HIV infected adults indicate that total IgE is also increased during the early stages of disease, and this elevation appears to be independent of CD4 counts and is not correlated with the levels of other immunoglobulins, suggesting an important role for IgE as a surrogate marker of disease progression Further research need to be exploited to bring out the exact role of IgE in HIV pathogenesis.

P1057

TB-IRIS is marked by acute phase response related elevation of LBP and IL-6

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Purpose/Objective: The immunopathogenesis of tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) is not well understood. The presence of elevated levels of IL-6 and C-reactive protein has been reported in TB-IRIS and are indicative of an acute phase response. Stimulation of the immune system by pathogen associated molecular patterns like lipopolysaccharide (LPS) leads to the production of IL-6 and acute phase proteins. Here we aimed to explore the acute phase response in TB-IRIS, in association with the cytokine storm and plasma markers related to LPS.

Materials and methods: We followed up 254 HIV+TB+ patients at Mulago Hospital in Kampala, Uganda of whom 53 developed TB-IRIS during antiretroviral therapy (ART). We compared 40 of these TB-IRIS patients with 45 HIV+TB+ patients from the same cohort who did not develop TB-IRIS (matched for age, gender and absolute CD4 T-cells). We analysed plasma levels of LPS, LPS-binding protein (LBP), sCD14, endotoxin core antibody (EndoCAb), intestinal-fatty acid binding protein (I-FABP, a marker of enterocyte damage), and 18 different cytokines before and after initiation of ART.

Results: LBP levels in TB-IRIS patients were significantly lower pre-ART ($P = 0.008$) and were sharply increased during TB-IRIS cases compared to HIV+TB+ controls ($P = 0.003$). No differences in sCD14, LPS, EndoCAb and cytokine levels were observed pre-ART between HIV+TB+ patients that did or did not develop TB-IRIS. During TB-IRIS however, higher levels of IL-1ra, IL-6, IL-7, IL-8, IL-10, G-CSF and IP-10 were detected compared to HIV+TB+ controls at comparable time points ($p \leq 0.027$). Surprisingly, I-FABP levels were significantly decreased during TB-IRIS and remained lower than in HIV+TB+ controls during follow-up ($P = 0.005$). No such differences were observed for LPS, sCD14 and EndoCAb. Multivariate analysis showed a central role for IL-6 in the acute phase response during TB-IRIS ($P = 0.001$).

Conclusions: Our data confirm a central role for IL-6 in the inflammation that develops during IRIS, while levels of LPS and EndoCAb suggested no additional effect of bacterial translocation from the gut. TB-IRIS is characterised by lower LBP levels pre-ART, followed by elevated levels during IRIS as part of the acute phase response. These findings suggest that LBP might be a useful marker for TB-IRIS.

P1058

The human leukocyte antigen-G 3'UTR 14-bp deletion is associated with poor survival in an HIV-1-infected Zimbabwean population

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Purpose/Objective: To examine the human leukocyte antigen G (HLA-G) 14-bp deletion polymorphism (rs16375) in relation to progression and survival among HIV-1-infected Zimbabwean individuals.

Materials and methods: A treatment-naïve cohort, including 150 HIV-infected and 158-HIV uninfected individuals, was genotyped for rs16375 using a competitive allele-specific PCR system. Survival among HIV-1-infected individuals followed for up to 4.3 years was compared between carriers of the +14-bp allele and -14-bp homozygous carriers by a log-rank test allowing for trend and by Cox proportional hazards regression analysis with adjustment for age and sex.

Results: The *HLA-G* homozygous -14/-14 genotype was associated with lower CD4 cell count ($P = 0.017$) and higher HIV-1 RNA ($P = 0.005$). Furthermore, the *HLA-G* homozygous -14/-14 carriers

had a higher mortality rate compared with non-carriers (hazard ratio = 1.9; $P = 0.04$, CI: 1.033−3.522); however, this difference was not statistically significant after adjustment for CD4 cell count and HIV-1 RNA (hazard ratio = 1.4; $P = 0.29$, CI: 0.753−2.578).
Conclusions: The *HLA-G* 14-bp deletion polymorphism is associated with higher viral load, more advanced progression, and increased mortality. The data suggest that high sHLA-G expression impairs the cytotoxic control of HIV.

P1059

The MHC-II transactivator CIITA is a viral restriction factor against HIV-1 replication

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Purpose/Objective: The MHC-II transactivator CIITA inhibits HIV-1 replication in human T cells by competing with the viral transactivator Tat for the binding to Cyclin T1 of PTEF-b.

Here we analyzed the anti-viral function of CIITA in a monocyte-macrophage model of HIV-1 infection, the U937 promonocytic *Plus* and *Minus* clones characterized by efficient or inefficient capacity to support HIV-1 replication, respectively. Recently, the *Plus* and the *Minus* phenotypes have been correlated to the absence or presence of the host factor TRIM22, respectively. Our purpose was to assess the functional relationships between CIITA and TRIM22.

Materials and methods: MHC-II/CIITA expression was assessed by FACS and QRT-PCR analyses. U937 *Plus* cells were stably transfected with CIITA vector by electroporation. Tat-dependent HIV-1 LTR transactivation was assessed by gene reporter assay. Exogenous CIITA and TRIM22 were co-immunoprecipitated in 293Tcells. *Plus*, *Minus* and *Plus*-CIITA cells infected with HIV-1 IIIB were monitored for RT activity over-time.
Results: U937 *Minus* cells express MHC-II molecules on the cell surface whereas *Plus* cells do not. This phenotype correlates with the expression of CIITA protein restricted to *Minus* cells. Importantly, we show that Tat-dependent HIV-1 LTR transactivation is reduced in *Minus* cells compared with *Plus* cells. The exogenous expression of CIITA did not induce TRIM22 transcription in *Plus* cells, whereas it was sufficient to inhibit Tat activity and to change the HIV-1 permissive phenotype of Plus cells to a non-permissive *Minus* 'like' phenotype.
Conclusions: We uncoupled the role of TRIM22 and CIITA in the inhibition of HIV-1 replication in monocytes cells. The transcriptional activity of Tat and HIV-1 productive infection were inhibited not only in TRIM22/CIITA-expressing *Minus* cells but also in *Plus* cells expressing exogenous CIITA. Thus, CIITA inhibits Tat-activity independently of TRIM22.

These findings demonstrate that CIITA has a dual function against HIV-1: it triggers the adaptive immune response by promoting viral antigen presentation and it acts as an endogenous restriction factor against viral transcription.

Poster Session: Inflammatory & Atopic Skin Disease

P1060

AGF inhibited a LPS-induced inflammatory response by blocking NF-kappaB activity in raw 264.7 cells

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Purpose/Objective: Inflammation is a system used by a host to defend against the presence of bacteria, viruses, or yeasts. Toll-like receptors (TLRs) in the plasma membranes of macrophages are activated when they recognize the molecular structure of a virus or bacterium. Lipopolysaccharide (LPS), an outer cell-wall component of Gram-negative bacteria, initiates an inflammatory process *via* TLR4. We investigated the effect of the extract of *Anethum graveloens* flowers (AGFs) on LPS-mediated inflammation in RAW 264.7 cells.
Materials and methods: The amount of NO production in LPS-induced macrophages was measured using Griess reagent. If AGF has a toxic effect on cells, one must distinguish such an effect from that of NO production, which also reduces the viability of LPS-induced cells. Hence, we compared cell cytotoxicity in RAW 264.7 cells incubated with AGF for 24 h using an EZ-Cytox kit. In addition, we investigated to determine whether the suppression of NO production was due to downregulation of iNOS expression. LPS-induced mRNA expression and the protein levels of iNOS were investigated in this experiment. In order to determine the effects of AGF on cytokines, we analyzed cytokine mRNA levels by RT-PCR. We further confirmed that the capability of AGF to activate the LPS-induced signal pathway involves downstream signal molecules such as MAPKs and Akt.
Results: The extract markedly suppressed nitric oxide generation in a concentration-dependent manner in LPS-stimulated RAW 264.7 cells. It inhibited inducible nitric oxide synthase (iNOS) and the mRNA expression of cytokines such as interleukin-1 beta and interleukin-6 in LPS-stimulated RAW 264.7 cells. It also inhibited iNOS protein levels in LPS-stimulated RAW 264.7 cells. In addition, AGF decreased the LPS-induced phosphorylation of mitogen-activated protein kinases in LPS-stimulated RAW 264.7 cells. AGF inhibited the phosphorylation of Akt, an upstream molecule of the nuclear factor kappa B (NF-kB) pathway, and thus inhibited NF-kB activity in LPS-stimulated RAW 264.7 cells.
Conclusions: These results suggest that AGF exerts an anti-inflammatory effect in LPS-stimulated RAW 264.7 cells by inhibiting iNOS expression and blocking the NF-kB pathway.

P1062

Cellular phenotypes implicated in reversal reaction in co-infected HIV/leprosy patients

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Purpose/Objective: The expanded availability of antiretroviral therapy (HAART) has added another layer of complexity to the understanding of HIV/leprosy pathogenesis. It has been reported that the initiation of HAART is associated with the development of reversal reaction (RR) in co-infected HIV/leprosy patients. However, the impact of HIV infection and HAART on the cellular immune response to *M. leprae* (ML) remains unknown. This study investigated the immunological profile of HIV/leprosy patients giving special attention to the cellular activation status and memory profile of CD4+ and CD8+ T cells.
Materials and methods: Twenty-five individuals were assessed: co-infected HIV/leprosy patients with RR (RR/HIV); leprosy patients with RR without HIV (RR); and healthy controls (HC). IFNg production in PBMC culture was analyzed by ELISPOT. T cell subsets were evaluated by flow cytometry for immune differentiation/activation markers. Skin biopsies were also evaluated for T cell subsets by immunofluorescence.
Results: IFNg production in non-stimulated (NS) cells from RR/HIV group was higher than in RR and HC groups. RR patients also presented high IFNg production in response to ML independently of HIV infection. Cellular activation in RR/HIV patients was increased in both CD4+ and CD8+ T cells in comparison to RR and HC group as reflected by the expression of CD38 and CD69. These activation markers were also increased in PBMC from RR/HIV patients after ML stimulus. Analysis of skin biopsies from RR/HIV patients also showed augmented expression of CD38 and CD69 co-localizing with CD4 and CD8 T cells. A higher frequency in central and effector memory CD8+ T cells in response to ML in RR/HIV patients was also observed. The production of granzyme B and perforin by effector memory CD8 T cells in response to ML was associated with an increase in the death of ML-infected monocyte cells.
Conclusions: These data suggest that CD38 expression in CD8 T cells might be utilized as a tool to identifying HIV/leprosy individuals at risk for RR. Besides this, an increase percentage of cytotoxic CD8 effector memory T cells could be an additional mechanism in mediating the appearance of RR in co-infected patients. Furthermore, the immune response to ML in RR/HIV patients appears to be restored during HAART therapy as indicate by the increase in IFNg production.

P1063

CINCA syndrome in an infant presenting with hydrocephalus

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Purpose/Objective: Chronic infantile neurological cutaneous and articular syndrome (CINCA) is a very rarecongenital autoinflammatory disease. It is characterized by neonatal onset urticarial-like rash, recurrent fever, central nervous system (CNS) involvement, and chronic arthropathy, peculiar facial and morphological features. Local and systemic manifestations of disease develop as a result of elevated IL-1b production which may be related to missense mutations within the gene encoding cryopyrin.
Materials and methods: We describe an infant with CINCA syndrome who developed hydrocephalus.
Results: A seven months old boy admitted with chronic urticaria and hydrocephalus. He was born by normal vaginal delivery following an unremarkable pregnancy. The parents were healthy and nonconsanguineous. He had attacks of urticarial rash beginning from birth, recurrent episodes of fever and progressive enlargement of head after 4 months. Blood tests showed leukocytosis, anemia, elevated acute phase reactants. Investigations for intrauterine infections and metabolic diseases were negative. Immunological workup did not reveal any congenital immunodefficiency. Cerebrospinal fluid (CSF) examination was compatible with chronic meningitis. Antibiotic treatment failed to alleviate the clinical symptoms. He wasdiagnosed as having CINCA on clinical grounds.
Conclusions: CINCA syndrome is a well-defined clinical condition but its early identification is often missed. The IL-1Ra (Anakinra) treatment has given good results in CINCA subjects. Pediatricians should be aware of this rare condition in order to detect it quickly and start the appropriate treatment as soon as possible, allowing to stop the disease progression to the severe degrees such as deafness and mental retardation.

P1064

Effects of topically applied rapamycin and/or mycophenolic acid on TNCB-induced atopic dermatitis-like skin lesions of NC/Nga mice

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Purpose/Objective: Our aim was to determine whether topically applied rapamycin and/or MPA is effective in AD-liked skin lesions of NC/Nga mice.

Materials and methods: Four per cent rapamycin and/or 1% MPA were applied to the 2-chloro-1,3,5-trinitrobenzene (TNCB)-induced AD-like skin lesions in NC/Nga mice 5 days a week for 2 weeks. The mice were divided into nine groups: non-treated, TNCB only, vehicle, 4% rapamycin, 1% MPA, 4% rapamycin and 1% MPA mixtures (Rapamycin: MPA = 2:8, 5:5 and 8:2 ratio) and 0.03% protopic. The clinical efficacy of drugs was evaluated by ear thickness and severity scores of skin lesions. Histological inflammatory changes and mast cell infiltration were evaluated by H&E and toluidine blue stain, respectively. The mRNA and protein expression level of IL-4 and IFN-γ in skin lesions was determined by quantitative RT-PCR and immunohistochemistry.

Results: Topical application of 4% rapamycin and/or 1% MPA significantly reduced the clinical skin severity and mast cell infiltration in the TNCB-induced AD-like skin lesions, compared with vehicle ($P < 0.05$). One percent MPA markedly reduced both IFN-γ and IL-4 mRNA expression level compared with vehicle ($P < 0.05$), whereas 4% rapamycin significantly reduced IFN-γ but not IL-4 mRNA expression level, compared with vehicle ($P < 0.05$). Our results demonstrate that topical rapamcyin and/or MPA suppress TNCB-induced AD-like skin lesions of NC/Nga mice by suppressing the local Th2 and Th1 response.

Conclusions: These findings suggest that rapamycin and/or MPA may be one of the promising topical therapeutic candidates for AD.

P1065

Eriodictyol inhibits mast cell degranulation through the inhibition of ceramide kinase

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Purpose/Objective: Mast cells are the principal effector cells involved in allergic response, through the release of histamine. Eriodictyol is a unique constituent of the painted maple (*Acer mono*) and yerba santa (*Eriodictyon californicum*). Pharmacological activities attributed to eriodictyol include the control of blood vessel permeability in arthralgia and fracture, as well as antioxidant and antimicrobial effects. However, the anti-allergenic activity of eriodictyol has not been evaluate We investigated the effect of eriodictyol on mast cell degranulation and on an allergic response in an animal model.

Materials and methods: *Passive cutaneous anaphylaxis (PCA) analysis.*

The PCA animal model is widely used to evaluate the localized mast cell-mediated allergic reaction *in vivo*. We tested the effect of eriodictyol on allergic response in the passive cutaneous anaphylaxis (PCA) reaction.

β-hexosaminidase release assay.

The RBL-2H3 mast cell line was established from a rat basophilic leukemia. We evaluated the release of preformed allergic mediators using β-hexosaminidase as a biomarker of degranulation in the mast cells and then measured cell viability. The release of β-hexosaminidase was first measured following stimulation of the IgE-Ag complex.

Measurement of ceramide.

We measured the ceramide levels in mast cells. Ceramide is a sphingolipid and an effector in proinflammatory processes. Accordingly, we tested the effect of eriodictyol on ceramide levels in IgE/Ag-stimulated mast cells using HPLC.

Results: We also investigated the effect of eriodictyol on expression of the CERK involved in calcium-dependent degranulation, and on ceramide activation by multiple cytokines. Eriodictyol suppressed the release of beta-hexosaminidase, a marker of degranulation, and the expression of interleukin IL-4 mRNA. Eriodictyol inhibited the expression of CERK mRNA, reduced ceramide concentration in antigen-stimulated mast cells and suppressed the passive cutaneous anaphylaxis reaction in mice in a dose-dependent manner.

Conclusions: These results suggest that eriodictyol can inhibit mast cell degranulation through the inhibition of ceramide kinase, and that eriodictyol may potentially serve as an anti-allergic agent.

P1066

Evaluation of platelet functions using impedance aggregometer

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Purpose/Objective: Platelets are playing a major role in primary haemostasis. Their main functions are adhesion, secretion, aggregation and procoagulant activity. Formation of a hemostatic plug in response to vessel wall injury requires functional platelets and defects platelet functionsdefined as 'Platelet Dysfunctions'. Chronic urticaria (CU) is a relapsing disease of skin, associated with itching of swelling skin and erythema, which lasts for 6 weeks or longer. Several studies indicate coagulopathy may be involved in CU etiology. The aim of this study was to investigate the platelet functions using impedance aggregometer method in CU patients.

Materials and methods: Patients diagnosed as 'CU' from dermatology outpatient clinic were enrolled to the study. Venous blood samples from 32 patients with CU as well as 26 healthy controls were collected. Platelet counts and functions were measured. Patients with hereditary/acquired angioedema, anaphylaxis, as well as urticaria-related or systemic vasculitis- patients were excluded from the study. It was confirmed that none of the patients received oral anticoagulant drug treatment, had any infection, thromboembolism, hepatic or cardiac disease or underwent any surgical treatment. Healthy subjects had similar age and gender distribution as well as the patient population. Statistical analysis was performed by using student's *t*-test. Platelet function analysis was performed as *in vitro* platelet activation and aggregation using impedance aggregometer method. Platelets were stimulated with ADP, ASP, TRAP and ristocetin agonists. Platelet aggregations were measured as 'Area Under Curve' (AUC).

Results: Response of platelets from CU patients to ADP ($P < 0.017$), TRAP ($P < 0.042$), ristocetin ($P < 0.028$) were decreased in comparison to healthy controls. Platelet counts ($P = 0.587$) and response to ASP ($P = 0.178$) did not differ in both groups.

Conclusions: Impedance aggregometer method is frequently used in determining prehaemostatic effects of antithrombotic therapies in clinical studies and in clinical practice. In this study platelet functions of chronic urticaria patients and healthy controls weremeasured with impedance aggregometer and different responses after stimulations of agonists were observed. The use of this methodology seems to be accurate and valuable in clinical practice.

P1067

Expression of histamine H4 receptor in human skin and amelioration of experimental acute pruritus using H4 receptor antagonist

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Purpose/Objective: Histamine is a potent mediator of itch in humans, yet histamine H1 receptor (H1R) antagonists have been shown to be of limited use in the treatment of pruritic diseases. Recently described histamine H4 receptors (H4R) are expressed in hematopoietic cells and have been linked to the pathology of atopic dermatitis and asthma. Recent studies have raised the possibility that H4R may be involved in pruritic responses. Here we investigated the expression of H4R in human skin and efficacy of the H4R antagonist on pruritus using experimental mice model of acute pruritus.
Materials and methods: Dermal tissue specimens were obtained from osteoarthritis patients, and immunofluoresence staining was performed to ascertain whether H4R is expressed in human epidermal tissues or dermal fibroblast cultures. Scratching behavior was induced by histamine (300 nmol), substance P (100 nmol) or serotonin (100 nmol) injected intradermally into the rostral part of the back of each mouse. Mice model of dry skin pruritus was created by topical application of distilled water following acetone/diethylether (1:1) mixture twice daily upon the shaved area for 5 days. Fexofenadine (30/60/150 mg/kg), a selective H1R antagonist and JNJ7777120 (3/10/30 mg/kg), a selective H4R antagonist were administrated orally.
Results: Immunohistochemical staining showed that K10-positive differentiated keratinocytes in the prickle cell layer and granular layer of epidermis strongly expressed H4R. In contrast, H4R expression was less in K14-positive proliferating keratinocytes in the basal layer. Cultured human dermal fibroblasts also express the H4R. The H4R antagonist JNJ7777120 significantly reduced histamine- and substance P-induced scratching behavior in a dose-dependent manner. Moreover, JNJ7777120 reduced dry skin-induced scratching behavior. However, the inhibitory effects of fexofenadine, a H1R antagonist, on pruritus of these models were much smaller than that of JNJ7777120. Neither fexofenadine nor JNJ7777120 showed reduction in serotonin-induced scratching.
Conclusions: Results of this study suggest that keratinocytes increase expression of H4R following differentiation. Moreover, as suggested in this study, histamine may have an involvement via the H4 rather than the H1R in histamine- and substance P-induced pruritus and dry skin-induced pruritus. The H4R antagonist may be useful for the treatment of H1R antagonist-resistant pruritus.

P1068

Hidradenitis suppurativa is characterized by a defect of Th17/Th22 CD4+ T cells and an infiltration of regulatory T cells in the skin

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Purpose/Objective: Hidradenitis suppurativa (HS) is a chronic, inflammatory skin disease. Medical treatments in use for HS (antibiotics and immunosuppressive agents) point out to a deregulated immune response against microflora. *In situ* antimicrobial defense requires a balance between inflammatory and protective T cell responses and their regulation through a Th17/IL-22/Treg axis. We investigated this pathway in the blood and the skin of HS patients.
Materials and methods: Analyses of the phenotype and functional profile of T cell populations in PBMCs from HS ($n = 17$) and healthy donors (HDs; $n = 11$) were performed *ex vivo* and following *in vitro* stimulation. Production of cytokines (IL-17, IL-22, IFN-g) and expression of transcription factors (T-bet, RORgt, Foxp3) were assessed using qRT-PCR, multiparametric flow cytometry and functional assays using ELISA and luminex technologies. *In situ*, T cell populations were characterized by Histo-immunochemistry (HIC).
Results: HS patients exhibited a higher frequency of CD4+IL-17A+(median 2%) and CD4+IL-22+(1.32%) in the blood as compared to HDs (0.85% and 0.53%, respectively) ($P < 0.05$). Frequency of $CD4^+$ IL22+ expressing CCR10, a skin homing receptor, was significantly increased in HS (15.3% as compared to 8.1% in HDs) ($P = 0.033$). There was no difference between groups in the frequency of $CD4^+$ $CD25^{high}FoxP3^{high}CD127^{low}$ Treg. Stimulation of PBMC with either flagelline, *staphylococcus aureus* or *candida albicans* led to a higher production of IL-17, IL-22 and IFN-g in HS patients as compared to HDs. Expression of CD3, IFN-g, and FoxP3 mRNAs was increased in the skin of HS patients ($n = 4$) as compared to HDs ($n = 5$). In contrast IL-22 and RORgt RNA expression was decreased in HS patients. HIC analyses confirmed an infiltration of Treg, but not $IL\text{-}17^+$ T cells in the skin of HS.
Conclusions: HS patients are characterized by a high frequency of proinflammatory functional memory Th17/Th22 T cells in the blood capable to respond to bacterial products. Despite expressing homing receptors, a lower frequency of these cells was noted in the skin of patients, in contrast, to a higher infiltration of Treg. These results raise several hypotheses such as a defect of local production of antimicrobial peptides, key triggers of T cell homing, leading to the chronic perpetuation of inflammation in skin lesions.

P1071

Relationship of interleukin-13 and interleukin-33 serum concentration and selected clinical and immunological parameters in patients with atopic dermatitis

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Purpose/Objective: Interleukin-13 (IL-13) coordinates the allergic inflammation process. It stimulates lymphocytes B to IgE synthesis, affects the differentiation's process and survival time of mastocytes and eosinophils. Interleukin-33 (IL-33) stimulates mastocytes, eosinophils, basophils and Th2 lymphocytes to secrete IL-13. The possible role of these cytokines in the pathogenesis of AD is indicated. To assess the relationship between serum level of IL-13 and IL-33 and severity of disease, extension of skin lesions and immunological parameters as total serum concentration of immunoglobulin E (total IgE), the amount of white blood cells and elements of leucogram in AD patients.
Materials and methods: The study involved 60 patients with AD (32 women, 28 men) aged of 18−54 years and 20 healthy volunteers of control group. AD was diagnosed according to criteria of Hanifin and Rajka. Serum concentration of interleukins was evaluated by immunosorbent assay (R&D, USA). The extension of skin lesions and SCORAD were rated in patients, total IgE in serum and the number of white blood cells with the elements of leucogram was determined.
Results: The mean concentration of IL-13 in AD patients' serum was 154.4 pg/ml (95% CI = 91−217) and IL-33 14.1 pg/ml (95% CI = 12.3−16). The mean concentration of IL-13 in healthy subjects

was 149.5 pg/ml (95% CI = 132.1−167) and was significantly lower ($P = 0.01$) and IL-33 18.4 pg/ml (95% CI = 16.2−20.7) and was significantly higher ($P = 0.01$) than in AD patients. The average extension of skin lesions was 33.4% (95% CI = 25.4−41.4) and the average severity of disease was 53.7 (95% CI = 49−58.3). The study found a statistically significant correlation between serum concentration of IL-33 and the extension of skin lesions ($P = 0.03$) and severity of disease ($P = 0.03$). The mean concentration of total IgE in serum was 2500IU/ml (95% CI = 843−4157). Relationship between serum concentration of interleukins and total IgE in serum did not show significant dependency. The evaluation of dependencies between the number of white blood cells, elements of leucogram and serum concentration of interleukins shows a statistically significant correlation with IL-13 and the percentage of lymphocytes ($P = 0.03$) and basophils ($P = 0.03$).

Conclusions: The mean concentration of IL-13 in serum is significantly higher and IL-33 is significantly lower in AD patients compared to healthy subjects. Serum concentration of IL-33 has a positive correlation with the extension of skin lesions and the severity of disease.

P1072
Role of the Aryl hydrocarbon receptor in skin inflammation

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Purpose/Objective: The Aryl hydrocarbon receptor (AhR) recognizes polycyclic aromatic hydrocarbons (PAHs), a family of structurally related environmental contaminants. Recently, AhR ligation was shown to amplify the developmental program of Th17 cells and to induce IL-22. By being in constant contact with the environment, the skin is exposed to multiple pollutants that are potential AhR agonists. Exposure to the best known AhR ligand, dioxin, for instance, leads to a severe skin disorder in humans. Taking into consideration the broad expression of AhR in the skin, which includes lymphocytes, dendritic cells and epithelial cells, we aim at defining skin immune responses in wild-type and AhR-deficient mice in order to assess the impact and integration of AhR activation on different cells in the skin.

Materials and methods: For this end we are making use of *in vivo* models of sterile injury (mechanical skin wounding), infection (*Candida albicans* skin infection) and immune-pathology (Imiquimod-induced psoriasiform skin inflammation). Parameters analysed included histological analysis of the skin and assessment of transcriptional changes, immune cell infiltration and *ex vivo* measurement of pro-inflammatory mediator secreted by resident and recruited skin cells. Additionally, the effect of dietary, endogenous and environmental AhR ligands on different cell types and its consequences for the development of skin inflammation are being assessed.

Results: The inflammatory reaction following simple mechanical skin injury was found to be stronger in AhR-deficient mice, which displayed an increased influx of neutrophils to the injury site. Similar results were obtained in the *Candida albicans* skin infection model, where the higher inflammatory milieu of the infected skin appeared to be detrimental for the infection clearance, as mice lacking AhR had higher fungal burden. Moreover, lack of AhR resulted in a more severe phenotype induced by the TLR7/8 agonist imiquimod, with increased thickness of the epidermis, neutrophils and inflammatory macrophage infiltration, and expression of key pro-inflammatory cytokines and chemokines.

Conclusions: Taken together, our results suggest that AhR signalling has a critical role in dampening immune processes in the skin. Future work will dissect the mechanisms underlying the stronger inflammatory response observed in AhR-deficient mice using cell type-specific AhR-deficient mice, to address what effect the lack of AhR in different cell types present in the skin has in different models of skin inflammation.

P1074
Th17 cells favor inflammatory responses while inhibiting collagen production by SSc fibroblasts

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Purpose/Objective: Th17 cells are augmented in Systemic Sclerosis (SSc), an autoimmune disease characterized by fibrosis of the skin and internal organs due to uncontrolled fibroblast activation. Our aim was to assess whether Th17 cells and IL-17A could modulate inflammatory and fibrotic responses in dermal fibroblasts from SSc and healthy individuals (HD).

Materials and methods: Fibroblasts were obtained from eight SSc skin biopsies and abdominoplasty pieces of 8 age/sex matched HD. Th17 cell clones were generated from the peripheral blood of HD upon enrichment of cells expressing CCR4/CCR6/CD161 and their cytokine production assessed by FACS analysis and multiplex beads immunoassay. MCP-1, IL-8, MMP-1 and type I collagen production was quantified in fibroblast supernatants by ELISA and RIA, and relative change in their transcription levels assessed by real-time PCR. IL-17 neutralizing antibody was used to confirm the specificity of the effects. Signaling events induced by IL-17A were investigated by western blotting and pharmacological inhibitors used to dissect the pathways involved.

Results: IL-17A increased MCP-1 ($P < 0.01$), IL-8 ($P < 0.01$) and MMP-1 ($P < 0.01$) production in a dose-dependent manner, while having no effect on type I collagen synthesis in 8 HD and 8 SSc fibroblasts at both protein and mRNA level. IL-17A induced the production of pro-inflammatory chemokines MCP-1 and IL-8 by triggering NF-kB and p38 signaling, while enhancing MMP-1 via activation of the JNK pathway. Supernatants of activated Th17 clones strongly enhanced MCP-1, IL-8 and MMP-1 production while inhibiting collagen synthesis. IL-17A neutralization proved the role of IL-17A in mediating Th17 effects. In clone supernatants, IL-17A had an additive/synergistic activity with TNF, as shown by IL-17A/TNF blockade. Consistently, IL-17A synergized with TNF in enhancing MCP-1, IL-8 and MMP-1 production when added to fibroblast cultures. Finally, TNF/IL-17 blockade in Th17 clone supernatants resulted in enhanced collagen production specifically in SSc fibroblasts.

Conclusions: Th17 cells elicit *in vitro* pro-inflammatory responses while limiting collagen production by fibroblasts. The increased Th17 cell number observed in SSc may impact on the inflammatory component of the disease and have rather a protective role against fibrosis.

P1075
The levels of cytokines, serum IgE and melatonin in children with atopic dermatitis with sleep disturbance

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Purpose/Objective: Patients with atopic dermatitis (AD) frequently reported disturbed sleep leading to impaired quality of life. The aims of

this study are to objectively measure the characteristics of their sleep disturbance, to determine the potential roles of scratching, cytokines, and melatonin, and to evaluate the impact of sleep disturbance on behavior.

Materials and methods: Forty-six AD patients and 32 healthy controls between 1 and 18 years old were enrolled. Subjective sleep quality is evaluated by questionnaire. Objective sleep parameters were determined by polysomnography and actigraphy. Serum levels of cytokines related to sleep (IL-1β, IL-4, IL-10, IL-6) and itch (IL-31 and INF-γ), serum total immunoglobulin E (IgE) levels, and urine melatonin sulfate levels were measured on the subsequent morning. The SNAP-IV and the Strength and Difficulties (SDQ) questionnaires were used to evaluate behavior.

Results: Subjective recognition of poor sleep quality was present in 60.9% of AD patients, compared with only 6.3% in controls. Objective measurements showed that sleep efficiency was lower in AD patients ($72.2 \pm 10.2\%$ versus $81.2 \pm 7.6\%$, $P < 0.001$). Sleep onset latency and wake time after sleep onset are longer in AD patients. Disease severity of AD was related to sleep efficiency ($r = -0.52$, $P < 0.001$) and movements in sleep ($r = 0.69$, $P < 0.001$). Total serum IgE levels were correlated with sleep efficiency ($r = -0.46$, $P = 0.001$). Morning urine melatonin sulfate level was higher in AD patients (87.7 ± 42.9 versus 71.1 ± 55.2 ng/ml, $P < 0.001$), and was correlated with sleep efficiency ($r = 0.41$, $P = 0.01$). Serum IL-1β, IL-4, IL-10, IL-6, IL-31 and INF-γ levels were not associated with sleep parameters. Lower sleep efficiency in AD patients is associated with higher oppositional defiant disorder score of SNAP-IV ($r = -0.47$, $P = 0.002$), and higher conduct problems score and total difficulties score in SDQ ($r = -0.42$, $P = 0.009$ and $r = -0.35$, $P = 0.027$, respectively).

Conclusions: Objective evaluation with polysomnography and actigraphy showed that poor sleep efficiency is common in AD patients, is associated with disease severity, and may have impact on behavior. Scratching movements, serum IgE, and melatonin might play a role in their sleep disturbance, and further studies are required to explore the mechanisms.

P1076

Truncated forms of IL-36alpha, beta and gamma cause mast cell activation and enhance the sensitivity of mast cells to stimulation via FcepsilonR1

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Purpose/Objective: IL-36α, β and γ (previously IL-1F6, IL-1F8 and IL-1F9) are IL-1 family members that signal via the receptor IL-1Rrp2 in complex with IL-1RAcP. Levels of IL-36α are increased in psoriatic skin, suggesting that the cytokine may play a role in the disease, and in support of this over-expression of IL-36α in keratinocytes causes skin inflammation in mice. Until recently, it has been difficult to study the IL-36 cytokines *in vitro* since the full length proteins are only active at high concentrations. It is now known, however, that N-terminal processing increases their potency a thousand-fold. It seems likely that the IL-36 cytokines are processed *in vivo* in a similar manner to IL-1α and β, producing more potent agonists. The truncated proteins have been shown to activate T cells and dendritic cells to produce cytokines and upregulate co-stimulatory molecules. Since mast cells are found at high numbers in the skin and are implicated in psoriasis and skin inflammation, we investigated whether IL-36α, β and γ can activate mast cells.

Materials and methods: Experiments were carried out *in vitro* using bone marrow derived mast cells (BMMCs).

Results: We find that BMMCs express the specific receptor for IL-36, IL-1Rrp2, and that the IL-36 cytokines cause bone-marrow derived mast cells to secrete IL-6, with IL-36β and γ being the most potent of the three. IL-36 increases the response of mast cells to stimulation via FcεR1, in a similar manner to that observed to occur with IL-33. Interestingly, BMMCs deficient in another IL-1 cytokine family receptor, ST2, are significantly less able to respond to IL-36α, β and γ. It is possible that ST2 is required for the expression of IL-1Rrp2 or IL-1RAcP in mast cells, or is involved in signal transduction after stimulation with IL-36α, β and γ.

Conclusions: These findings may be of particular relevance in disease settings where IL-36 is expressed, for example in psoriatic legions. The cytokine could act to cause mast cell pro-inflammatory cytokine secretion in addition to enhancing the response of mast cells to antigen, therefore exasperating inflammation.

Poster Session: Inflammatory Bowel Diseases

P1079
Allogeneic mesenchymal stromal cells (MSC) transplant in experimental inflammatory bowel disease: modulation of gut inflammation and IL-17 dependent responses

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Purpose/Objective: Inflammatory Bowel Diseases (IBD) is chronic inflammation of the intestinal mucosa, with uncontrolled Th1 and Th17 responses. Although there are current therapies, no treatment is at present fully effective. Since Mesenchymal Stromal Cells (MSC) is multipotent, regulatory and immunosuppressive cells, they emerge as a therapeutic option for various immune disorders. The objective was to evaluate the role of allogeneic MSC transplant in the treatment of experimentally induced IBD.
Materials and methods: IBD was induced in BALB/c mice with intrarectal injection of Trinitrobenzene Sulfonic Acid (TNBS). MSC were isolated from bone marrow of C57BL/6 mice, cultured and phenotyped for characterization. IBD mice received intraperitoneal injection of MSC 24 h after disease induction and were euthanized 3, 7 and 14 days later for sample collection, besides clinical evaluation of gut inflammation.
Results: MSC cultures in 4th passage showed characteristic markers, such as CD105, CD73, CD44, CD90, CD29, although CD45, CD31, CD34 and CD11b were also found, indicating some contamination of the MSC culture with cells of hematopoietic lineages. Even though, IBD mice treated with MSC presented gain of weight and reduced clinical score compared to not treat mice. There was an apparent increase in eosinophil and reduction in neutrophil influx in treated animals' colon. Regarding intestinal cytokines, we found an increase of IL-10 and IL-12 on day 3 together with a strong reduction in IL-17 after MSC treatment. In histological analysis, the percentage of goblet cells was higher in treated group than in not treated mice, showing restoration of intestinal morphology by MSC transplant.
Conclusions: MSC are able to modulate gut inflammation and IL-17 responses in experimental IBD model. Thus, MSC treatment of IBD may be a novel therapeutic tool aimed at modulating mucosal immune responses without apparent or undesirable adverse effects.

P1080
Anti-TNF therapy response in patients with inflammatory bowel disease is associated with T cell expression of CD25 and TNF receptor 2

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Purpose/Objective: Anti-tumor necrosis factor (anti-TNF) agents are effective treatment options for certain patients with corticosteroid dependent or refractory inflammatory bowel disease (IBD). However, the cellular mechanisms behind anti-TNF treatment leading to therapy response are still incompletely known.
Materials and methods: Blood samples were obtained before first treatment (visit 1) and 2 weeks post treatment (visit 2) from patients who commenced anti-TNF treatment. The disease activity was assessed by the validated Mayo score and Harvey-Bradshaw Index (HBI). Response was defined as a decrease in Mayo score or HBI with ≥3. The immunological effects of anti-TNF therapy were studied on freshly isolated cells and cells stimulated *ex vivo* with influenza vaccine, using flow cytometry.
Results: We have included 23 IBD patients (6 CD and 17 UC) into the study. Sixteen patients responded to the anti-TNF therapy, whereas seven patients did not respond. A reduction in the proportion of circulating $CD25^+$ CD4+ T cells [23.9 (5.3−49.6) versus 20.7 (4.4−38.5), $P = 0.003$] among freshly isolated cells was detected in responders when comparing the first visit (pre treatment) and the second visit (2 weeks post treatment). In contrast, non-responders showed an increased frequency of $CD25^+$ CD4+ T cells [14.3 (6.4−33.4) versus 25.3 (6.6−45.2), $P = 0.03$] when comparing visit 1 and 2. Levels of $FoxP3^+$ CD4+ T cells and $AnnexinV^+$ CD3+ T cells were similar between both patient groups. Also, there was no difference in expression levels of TNF receptor 1, TNF receptor 2 (TNFR 2) or membrane bound TNF on circulating T cells or monocytes. To compare the T cell phenotype of therapy responders and non-responders before anti-TNF treatment, cells from visit 1 (pre treatment) were stimulated with influenza vaccine in the presence or absence of anti-TNF antibodies. The effect of anti-TNF is shown as the reduction of CD25 and TNFR2 expression relative to cells cultured without anti-TNF. Results showed that anti-TNF induced a greater reduction of both $CD4^+$ CD25+ (35.1% (14.3−87.4) versus 21.2% (-29.6−39.4), $P = 0.03$) and CD3+TNFR2+ (51.6% (30.2−79.7) versus 20.3% (4.1−29.4), $P = 0.002$) T cells in therapy responders as compared to non-responders.
Conclusions: This study indicates that successful anti-TNF therapy induces a reduced activation of T cells *in vivo*. Moreover, the expression of CD25 and TNFR2 on *in vitro* stimulated T cells in the presence of anti-TNF antibodies before treatment start may predict the therapeutic response.

P1081
Colonic Ileus after intestinal surgery depends on CCR2

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Purpose/Objective: Dendritic cells (DCs) are very potent in inducing adaptive immune responses against pathogens, but also cause intestinal autoimmune diseases like colitis. It has been shown previously that the most severe complication after abdominal operation, the post operative Ileus (POI), depends on macrophages (MPs) and memory T helper type 1 (mTh1) T cells, which are initially activated by DCs. However, the specific POI-inducing DC-subset and the role of chemokine receptors in POI-induced DC-migration is still unclear.
Materials and methods: After opening the peritoneal cavity POI was induced in $CCR2^{-/-}$, $CX3CR1^{+/-}$ and C57BL6 mice by manipulating the small bowel with moist cotton applicators once from the oral to aboral direction. After 24 h small bowel function was assessed through feeding FITC labeled Dextran and measuring its bowel progression 1.5 h later. Large bowel function was examined by inoculation of a bead into the colon and measurement of the excretion time. Cell numbers were determined using flow cytometry.
Results: We found that the presence of CX3CR1-expressing DCs after abdominal surgery was dependent on CCR2, but lack of such DCs did not improve POI in manipulated small bowel segments. However, CCR2-deficiency improved gut motility in the non-manipulated large bowel. Such improved gut motility was not due to reduced dissemination of POI by mTh1-cells, because recirculation of mTh1 cells was

not affected. Notably, F4/80$^+$ CD11c$^-$ MPs were significantly reduced in the large bowel of manipulated CCR2-deficient mice.
Conclusions: These findings indicate that CCR2-dependent DCs are dispensable for local POI-development and suggests that CCR2-dependent MPs might contribute to POI within the non-manipulated large bowel.

P1082
Deficient production of reactive oxygen species leads to a chronic DSS-induced colitis in Ncf1-deficient B10.Q mice

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Purpose/Objective: Chronic Granulomatous Disease (CGD) is a genetically heterogeneous immunodeficiency disorder caused by deficiency in oxidative burst, resulting in increased susceptibility tobacterial and fungal infections. CGD is caused by mutations in the phagocyte NADPH oxidase complex, the enzyme which generatesoxygen radicals. A common clinical complication in CGD is chronic intestinal inflammation. As in humans, Ncf1 mutation leads to the lack of reactive oxygen species (ROS) in B10.Q mice, increasing their susceptibility to autoimmunity and infection. We used these mutant mice to study how the lack of ROS influences DSS-induced colitis, its transitional recovery and a second colitis induction.
Materials and methods: In the study were usedhomozygous Ncf1 mutatedand B10.Q (Wt) mice. Colitis was induced by oral administration of 3% (w/v) DSS. The induction protocol consisted of 7 days of treatment with DSS followed by 7 days of resting on normal water and finally a second cycle with DSS. Micewere sacrificed at the end of each time point. During the experiment we monitored clinical scores of colitis, collected blood for serum cytokine quantification using CBAs and phenotiping by flow cytometry. We also collected colons at each time point for HE and immunohistochemical (B220, MAC1, CD3) evaluation.
Results: The clinical and histological analyses revealed that Ncf1 mice had a more severe disease with a weaker recovery and signs of a chronic colitis. Cytokines quantification showed that both groups had similar Th1/Th17 behavior in the acute phase, but with earlier higher levels of IL-2, IL-6, IL-17, IL-21, IFNγ and IL-10 in Ncf1. In the 2nd induction Ncf1 reduced IL-21 compared to Wt. Phenotipe data show a marked Th1/Th17 response in both groups. Ncf1 had more CD4 and CD8 T cells expressing CXCR5 and CD69, NK cells expressing CD107a, Tregs expressing CTLA4 and CXCR5 and B cells expressing CXCR4. In the acute phase and at the 2nd induction Ncf1 had more central memory andeffector CD8 T cells and less Treg compared to Wt. Compared to Wt, Ncf1 circulating monocyte pool evolved from having fewer mature CD11b$^+$Ly6c^{low} at T0 towards an accumulation of CD11b$^+$Ly6c^{hi} at T1 and T2, and finally more CD11b$^+$Ly6c^{low} at T3.
Conclusions: The clinical scores, the immunohistopathology of colon biopsies, the quantification of serum cytokines and the flow cytometric analysis of peripheral blood mononuclear cells subsets suggest that ROS absent in Ncf1-deficient B10.Q mice leads to an aberrant inflammatory state leading to the development of a chronic colitis similar to seen in CGD humans.

P1084
Dissecting the disease associated functions of S1PR/SPHK axis in DSS-induced colitis in mice

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Purpose/Objective: Ulcerative colitis (UC) is the most common type of inflammatory bowel disease (IBD) afflicting humans. It causes chronic inflammation and the development of ulcers of the large intestine. The aetiology of colitis is not clearly well defined. Dextran Sulphate Sodium (DSS)-induced colitis in mice is a well studied model for human UC. In the present study, we have specifically dissected the role of Sphingosine-1-Phosphate Receptor (S1PR)/Sphingosine Kinase (SPHK) axis in the development of DSS colitis using a metanalysis approach and DSS-colitis model using both SPHK1$^{-/-}$ and SPHK2$^{-/-}$ mice.
Materials and methods: Meta-analysis of high-throughput genomics data to decipher the role of S1PR/SPHK axis in DSS colitis in mice. The raw Affymetrix CEL files downloaded from the Gene Expression Omnibus (GSE22307) were analysed using the Genespring GX11.5 (Agilent, USA). The statistically significant gene list was filtered based on the standard twofold cut-off compared with the Day 0 expression values.

DSS colitis.

C57BL/6j mice were purchased from Harlan Olac. SPHK1$^{-/-}$ and SPHK2$^{-/-}$ mice were all on the C57BL/6j background. Mice were housed in specific-pathogen-free conditions in the Central Research Facility and the experiments were conducted in accordance with the respective animal experiment guidelines. For acute colitis induction, Wild-Type (WT), SPHK1$^{-/-}$ and SPHK2 $^{-/-}$ male mice were given 3.5% (weight/volume) DSS (molecular weight 36–50 kDa; ICN Biomedicals, Aurora, OH, USA) in their drinking water from day 0 for consecutive 7 days. The control mice were given only normal drinking water. The body weight, stool consistency, and rectal bleeding were monitored daily using the modified method of Cooper and colleagues. All the mice were sacrificed on day 7. The colons were dissected and properly cleaned. Sections were taken for histology and serum for multiplex cytokine assay.
Results: The meta-analysis of the high throughput genomics data showed that S1PR3 and SPHK1 were the early induced genes in the colonic epithelia of the DSS-treated WT mice. However, both SPHK1$^{-/-}$ and SPHK2$^{-/-}$ mice were protected from the DSS-induced body weight loss, pathological changes in the colon as evidenced by colon length and histological score and attenuated proinflammatory cytokine profiles when compared with DSS-treated WT mice.
Conclusions: The S1PR/SPHK axis in the colonic epithelium plays a key role in the development of DSS-colitis. Moreover, both SPHK1 and SPHK2 are important for the effective induction and exacerbation of DSS-colitis.

P1085
Elevated expression of TH17-associated cytokines in the colon of active ulcerative colitis

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Purpose/Objective: The functions of TH17 cytokines in IBD remain controversial. Genes involved in the downstream signaling of IL-23,

the cytokine that promotes and sustains TH17 differentiation, have been shown to mediate susceptibility to UC. However mouse models of IBD have demonstrated both protective and pathogenic effects of TH17 associated cytokines. This study measured TH17 and TH1 cytokines in matched peripheral blood and colonic biopsies to determine whether there is a detectable alteration in cytokine patterns in active UC compared to those in remission and healthy controls.

Materials and methods: Matched peripheral blood and colonic biopsies were studied in 8 patients with active UC, 8 patients with inactive UC and 10 healthy controls using multi parametric flow cytometry. Disease activity was defined by standard clinical criteria (Mayo score). Mucosal mononuclear cells (MMCs) were isolated by collagenase II digestion followed by mechanical disruption then cell straining. Cytokine staining performed on PBMCs and MMCs after stimulation with SEB. We measured the proportion of CD4 that expressed TH1 and TH17 cytokines as well as amount of cytokine secreted by CD4 T cells using the median fluorescence intensity (MFI).

Results: UC patients in remission had significantly higher CD4 percentages in the peripheral blood compared to those with active disease (81%, 55%, $P = 0.01$). The proportion of $CD4^+$ CD161+ was significantly reduced in PBMCs (active UC = 7%, control = 9%) and MMCs (active UC = 54%, control = 63%) of patients with active UC compared to controls. The MFI of IFNγ and TNF on mucosal CD4 T cells was significantly raised in active UC compared to controls ($P = 0.04$, $P = 0.01$). The MFI of IL-17a on mucosal CD4 T cells was also significantly raised in patients with active UC compared to controls ($P = 0.03$). A significantly increased number of MMC CD4 T cells were found to express IL-22 in active UC compared to controls (1.00%, 0.75%, $P = 0.01$).

Conclusions: Active UC is associated with increased expression of inflammatory TH1 and TH17 cytokines. Biological agents to inhibit inflammatory pathways may have a role in the therapy of UC.

P1086

Evaluation of the piroxicam-accelerated interleukin-10 knocks out mouse — as a model of human inflammatory bowel disease

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Purpose/Objective: The pathogenesis of inflammatory bowel disease (IBD) is still poorly understood, but it is believed to result from a multifactorial condition, where genetic and environmental factors play an interrelated role, leading to a breakdown of the intestinal homeostasis and an excessive immune response against the commensal microflora. The aim of this project is to evaluate whether the piroxicam-accelerated colitis interleukin-10 knock out mouse (PAC IL-10 k.o.) could function as a tool in the preclinical research and development of new therapeutics against IBD.

Materials and methods: The PAC IL-10 k.o. model was evaluated by clinical manifestations and the immune response was characterised by haematology, histopathology, colonoscopy, ELISA and FACS analysis. Qualification was performed by examining the efficacy of treatment with biological therapies for IBD (anti-TNFα and anti-IL-12/23p40). Also, the mice were treated with an antibiotic to determine the role of commensal bacteria in disease progression.

Results: The PAC IL-10 k.o. model developed a pronounced colitis immediately after the start-up of piroxicam administration, with synchronised weight loss, diarrhea and blood in stools. Clear signs of chronic colitis were present even 2 weeks after termination of piroxicam. Acute phase proteins and granulocytes were significantly elevated in the blood compared to IL-10 k.o. controls and histological evaluation revealed hyperplasia and marked infiltration of mononuclear cells and neutrophil granulocytes in the mucosa and submucosa. Disease progression was significantly reduced when treated prophylactically with neutralising monoclonal antibodies against IL-12/23p40 and TNFα, as well as ampicillin.

Conclusions: The presented data show that the PAC IL-10 k.o. mouse model may be useful as an *in vivo* model of human IBD, since the model resembles the corresponding human condition in many aspects.

P1087

GM-CSF modifies monocytes to develop anti-inflammatory properties that are beneficial in Crohn's disease

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Purpose/Objective: Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) showed clinical response and remission in patients with active Crohn's disease (CD). Since GM-CSF has pleiotropic effects on monocytes, which represent the exclusive source of macrophages in inflamed intestinal mucosa we characterized GM-CSF-treated mouse monocytes *in vitro* and analyzed their function *in vivo* in a mouse model of chronic DSS induced colitis.

Materials and methods: Mouse bone marrow-derived monocyte precursors were treated for 48 h with GM-CSF *in vitro*. Phenotypical changes were assessed by qRT-PCR and flow cytometry. Various functional properties were evaluated: mixed lymphocyte reactions, phagocytosis, adherence, cytokine production and reactive oxygen species (ROS) production. Therapeutic effects of GM-CSF-treated monocytes were assessed in a model of chronic colitis that was induced by repeated oral administration of DSS (2%). Monocytes were administered i.v. prior to start of final DSS treatment cycle and their subsequent immunomodulatory functions were evaluated *in vivo* by clinical monitoring (e.g. body weight), histology, immunohistochemistry and expression of inflammatory markers by qRT-PCR. The distribution of injected monocytes in the intestine was measured by *in vivo* imaging.

Results: GM-CSF-treated monocytes expressed significantly higher levels of anti-inflammatory molecules on mRNA and protein levels (e.g. IL1-Ra, IL4-Ra, CD121b and ARG1) e*x vivo*. GM-CSF induces ROS production but reduces phagocytosis and adherence in monocytes. Mice treated with GM-CSF stimulated monocytes showed resistance in chronic colitis with diminished weight loss compared to control mice. (Histo-) Pathology showed less inflammatory infiltration, ulceration, and colon shrinkage. Additionally, proinflammatory mediators IL-1, IL-6 and TNF-a were decreased during the course of colitis. GM-CSF-treated monocytes enter the intestine in significantly higher number and persisted longer compared to control monocytes in DSS treated mice.

Conclusions: Our results indicate that the beneficial effects of GM-CSF in CD may be in part due to the induction of monocytes with anti-inflammatory properties.

P1088

HDAC dependent regulation of the IL-6/STAT3 pathway during T helper cells activation

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Purpose/Objective: Histone modifications represent a promising new approach in cases where cell functions are to be modulated as in

autoimmune diseases or cancer. While several histone deacetylase (HDAC) inhibitors are currently in clinical cancer studies, we demonstrated an additional anti-inflammatory potency in murine colitis models. Here we describe a possible cellular mechanism for this effect.
Materials and methods: Murine naïve T helper cells were isolated via magnetic cell sorting and macrophages were derived from bone marrow (BMMΦ). T cells were stimulated using coated anti-CD3/CD4 antibodies, macrophages via LPS. Cells were analysed using flow cytometry, cytometric bead array or western blot. Acute DSS colitis was performed.
Results: In the presence of ITF2357, the generation of FoxP3$^+$ cells from naïve T helper cells could be enhanced, the polarization to the pro-inflammatory Th17 cells suppressed. In parallel, we demonstrated a dose-dependent downregulation of the IL-6 receptor on naïve CD4 T cells treated with ITF2357. This effect could be observed on the mRNA expression level and on the protein level via flow cytometry. These results were confirmed in murine colitis models, where the IL-6R expression was diminished on naïve T cells within the lymphnodes, paralleled by a significant reduction of Th17 cells in the lamina propria of ITF2357-treated animals. Consequently, HDAC inhibition resulted in a reduced amount of activated/phosphorylated STAT3 in T cells identifying the IL-6/STAT3/IL-17 pathway as an important target of HDAC inhibitors.

In parallel, ITF2357 treatment of BMMΦ leads to a dose-dependent down regulation of TNFα, IL-6 and IL-12p70 secretion by BMMΦ. TLR4-dependent IL-6R upregulation was significantly impaired by ITF2357, while expression of the signaling transducer CD130 was unchanged. ITF2357 reduced the ability of antigen-specific, MHC-II-dependent T cell activation.
Conclusions: The present study demonstrates that inhibition of HDAC exerts an anti-inflammatory potency by modulation in T cell polarization directly, but also via affecting macrophage differentiation, leading to impaired IL-6 signalling, reduced T cell activation, thus representing a novel therapeutic approach for chronic (intestinal) inflammation.

P1089

Identification, charactarization and epitope mapping of gamma gliadin, a major wheat antigen in celiac disease

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Purpose/Objective: Approximately 1% of the population suffers from celiac disease (CD), an inflammatory disease of the small intestine elicited by wheat ingestion. Affected patients mount T cell responses and IgA antibody responses to several antigens belonging to the gliadin-containing fraction of wheat but so far no recombinant wheat antigen specifically recognized by CD patients has been produced. Aim of this study was the biochemical, molecular and immunological characterization of recombinant wheat antigens specific for CD.
Materials and methods: Gliadins were fractionated into sub-fractions using ion-exchange chromatography and their antibody reactivity to patients sera was analysed by western blot and ELISA. The disease specific antigens in the sub-fractions were identified by mass spectrometry and N-terminal sequencing of the individual protein bands. Recombinant antigens were generated in bacterial expression system. Overlapping peptides covering the entire sequence of the recombinant antigen was synthesised and IgA/IgG epitope mapping was performed by ELISA. The secondary structure of the recombinant antigen was performed using circular dicroism.
Results: We developed a method for separation of gliadins by ion-exchange chromatography and identified disease specific antigens in the gliadin sub-fractions by studying their reactivity to serum IgA from clinically well defined CD and non-CD patients. Through mass spectrometry and N-terminal sequencing we identified the proteins in the sub-fractions and showed that the most relevant IgA-reactive CD-specific antigens belong to the class of gamma gliadins. Based on the sequences identified by mass spectrometry we cloned the complete cDNA sequence of a gamma gliadin (GG1) for recombinant expression and purification. Secondary structure of recombinant GG1 was analysed by circular dichroism. Recombinant GG1 was successfully purified and showed highly specific IgA reactivity with sera from CD patients. IgA and IgG epitope mapping studies with synthetic peptides revealed a major immunodominant regions located at the N-terminus of the protein. Circular dichroism analysis showed that the antigens were folded.
Conclusions: Recombinant GG1 should be useful for characterizing the immune response to wheat antigens and to develop diagnostic and therapeutic strategies for CD.

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P1090

IL-1 mediates intestinal inflammation by promoting the accumulation of IL-17A secreting innate lymphoid cells and CD4+ Th17 cells

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Purpose/Objective: Although very high levels of Interleukin (IL)-1β are present in the intestines of patients suffering from Inflammatory Bowel Diseases (IBD), little is known about the contribution of IL-1β to intestinal pathology. We aimed to define the role of IL-1β in driving innate and adaptive pathology in the intestine.
Materials and methods: We assessed the roles of IL-1β in complementary mouse models of inflammatory bowel disease, mediated by either innate or adaptive immune activation.
Results: We show that IL-1β promotes innate immune pathology in *Helicobacter hepaticus*-triggered intestinal inflammation by augmenting the recruitment of granulocytes and the accumulation and activation of innate lymphoid cells (ILC). Using a T cell transfer colitis model, we demonstrate a key role for T cell-specific IL-1 receptor (IL-1R) signals in the accumulation and survival of pathogenic CD4$^+$ T cells in the colon. Furthermore, we show that IL-1β promotes 'type-17' responses from CD4$^+$ T cells and ILC in the intestine and we describe synergistic interactions between IL-1β and IL-23 signals that sustain innate and adaptive inflammatory responses in the gut.
Conclusions: Our data identify multiple mechanisms through which IL-1β promotes intestinal pathology and suggest that targeting IL-1β may represent a useful therapeutic approach in IBD.

P1093

Interaction between *Trichinella spiralis* infection and inflammatory colitis: novel immunological concepts

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Purpose/Objective: The aim of this study was to gain insight about time-related interaction between intestinal nematode infection and inflammatory colitis in an effort to ameliorate experimentally induced colitis using a model for ulcerative colitis, and to explore the underlying immunoregulatory mechanism of *Trichinella spiralis* infection.
Materials and methods: Mice used were divided into four groups: group I: infected with *Trichinella spiralis*; group II: infected with *Trichinella spiralis* then subjected to induction of colitis; group III: subjected to induction of colitis and group IV: subjected to induction of colitis followed by *Trichinella spiralis* infection. Mice were sacrificed at 2th and 4th weeks post-colitis. Assessment of colitis was done by histopathological examination, and determination of pentraxin 3 level in the colon. Immunohistochemistry was done for identification of T regulatory Foxp3-expressing cells.
Results: It was evident that *T. spiralis* infection ameliorated the severe inflammation induced by acetic acid. The amelioration was more pronounced when *T. spiralis* infection preceded the induction of colitis. Mean pentraxin 3 values were significantly lower in case of colitis with Trichinella infection as compared to negative control or colitis group at different experimental periods. Regarding the immunohistochemical staining of T regulatory cells, the highest score of positivity was detected in group I (*T. spiralis* alone) and the least score was in group III (acetic acid induced colitis) with the other two groups inbetween.
Conclusions: *T. spiralis* regulatory mechanism can improve the inflammation of colon through the 'inflammatory regulatory' axis. Finally, it would be of great importance to apply these results in the development of new therapeutic approaches for treatment of ulcerative colitis.

P1094

KIR-mediated NK education mediates KIR-associated Crohn's disease susceptibility

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Purpose/Objective: Natural Killer (NK) cells are innate effector lymphocytes in the host response to infections and tumors through a variety of activating and inhibitory receptors. NK cells require education by self-human leukocyte antigen (HLA) class I molecules through Killer cell Immunoglobulin-like receptors (KIRs) to gain proficient responses. Yet, it remains unknown whether and how KIR-mediated NK education contributes to chronic inflammation in humans.
Materials and methods: First, in healthy subjects bearing the simplified AA KIR haplotype, NK subsets expressing KIR2DL3 and 3DL1 were analyzed for cytokine production by single cell barcode chip [SCBC, Ma, C, *et al. Nat Med* (2011)]. Second, genetic distribution of HLA-C1 and HLA-Bw4, the respective ligands for KIR2DL3 and KIR3DL1, in AA haplotype Crohn's Disease (CD) patients were analyzed by Chi square test. Third, CD patient NK culture media was profiled for cytokine production at the bulk level using multiplex ELISA system, and at the single cell level using SCBC. Lastly, an NK coculture assay was performed to test their effect on antigenic CD4+ T cell activation and mechanism of action.
Results: In this study of subjects bearing the simplified AA KIR haplotype, we show that KIR education enables NK cells to promote $CD4^+$ T cell proliferation by eliciting expression of multiple pro-inflammatory cytokines and chemokines. Using the SCBC microfluidic platform, we show that the genetically educated NK subset was highly polarized towards robust production of cytokines at the single-cell level. The strongly-educating KIR-ligand pair (KIR2DL3 and homozygote HLA-C1) was enriched in CD patients, and NK cells from patients with this genotype had distinct secretion profiles compared to NK cells from weakly educated patients. Autologous NK-T cell co-culture demonstrated that strong KIR education permitted NK augmentation of $CD4^+$ T cell proliferation *via* secretion of pro-inflammatory cytokines.
Conclusions: Collectively, these results extend our understanding of the functional consequences of NK education, and reveal the unappreciated capacity of NK cells to produce a wide spectrum of immune mediators that modulate the $CD4^+$ T cell activation threshold. These findings offer a biologic basis for the correlation between NK education and KIR-associated susceptibility to CD and other chronic inflammatory syndromes.

P1095

Mononuclear phagocytes in steady-state and colitis

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Purpose/Objective: There is increasing evidence that antigen-presenting cells are key determinants in shaping both innate and adaptive immune responses in the gut, thereby contributing to intestinal homeostasis. Recently, two major populations of intestinal mononuclear phagocytes have been identified on the basis of the differential expression of the integrin subunit CD103 and the fractalkine receptor CX_3CR1. The aim of this study is therefore to understand how these specific cellular subsets contribute to the pathogenesis of inflammatory bowel diseases.
Materials and methods: To address the function of colonic LP mononuclear phagocytes during intestinal inflammation, we utilized a mouse model of *Helicobacter hepaticus*-induced colitis combined with anti-IL10R treatment in CX_3CR1-GFP knock-in reporter mice.
Results: Upon infection, large numbers of antigen-presenting myeloid cells were rapidly detected in the colonic lamina propria and mesenteric lymph nodes, and persisted during the course inflammation. Detailed analysis of these cells revealed the primary accumulation of an activated inflammatory monocyte population expressing $MHCII^+$ $CD11c^{het}$ $Ly6C^{hi}$ CX_3CR1^{int} as well as the FCgReceptor IV (CD64), and producing TNFa, IL12p40 and iNos.
Conclusions: We therefore suggest that $CD64^+$ marks an activated subset of infiltrating CX_3CR1^{int} mononuclear phagocytes that produce colitogenic cytokines and perpetuate intestinal inflammation.

P1096

Neuronal CGRP and TNF alpha co-regulate each other in a mouse model of parasitic infection in the gut

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Purpose/Objective: Background: Neuropeptides have been associated with immune functions in many inflammatory models. In this study we examined the relationship between the sensory nociceptive neuropeptide calcitonin gene related peptide (CGRP) and the potent

inflammatory cytokine tumor necrosis factor alpha (TNFα) during a chronic helminth infection. CGRP has been shown to down-regulate TNFα production from macrophages *in vitro* whilst TNFα attenuates CGRP release from sensory neurons *in vitro*. Given the role for CGRP in mediating neuroinflammation, we wanted to study this interaction *in vivo* during an infectious challenge in the gut.
Materials and methods: Methods: CGRP and its receptor antagonist, hCGRP $_{8-37}$, were administered *in vivo* between days 14 and 21 post *Trichuris muris* infection in BALB/c and AKR mice. Additionally, a TNFα inhibitor, Infliximab, was administered *in vivo* in chronically infected AKR mice at day 35 p.i.
Results: Results: : During infection, an inverse correlation between CGRP and TNFa was observed. Furthermore, CGRP treatment *in vivo* reduced TNFα expression in both AKR and BALB/c mice during infection, implying a negative regulation. However, when TNFα was inhibited *in vivo* with infliximab, CGRP levels reduced in the colon, suggesting that CGRP may respond to local TNFa downregulation.
Conclusions: Conclusion: Our study shows for the first time that CGRP and TNFα inversely correlate with each other during gut inflammation *in vivo*. This demonstrates a role for CGRP in the immune response during parasite infection. Whether this happens directly or indirectly is the subject of further work.

P1097
Phagocytosis of *Escherichia coli* by human gamma delta T cells: a possible role in the pathogenesis of Crohn's disease

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Purpose/Objective: The aetiology of Crohn's disease (CD), although unknown, is generally accepted as being multifactorial. One of the factors that appear to be instrumental in disease initiation/progression is the immunological response to the gut enteric microbiota. We have previously shown that in cases of CD, mucosal associated *E. coli* are able to penetrate into the lamina propria and survive within macrophages. In this present study we have not only confirmed this observation but in addition, using immunofluorescence staining (IF), identified *E. coli* associated with a population of $\gamma\delta$ T cells in biopsies of patients with Crohn's disease. This would be consistent with previous literature that has indicated the role of $\gamma\delta$ T cells as APC's.
Materials and methods: Patients with CD were identified using the Montreal criteria, none of whom were on immunosuppressive or biologic therapy at the time of study. Controls were selected from a colorectal cancer screening population who had a normal bowel habit, no abdominal pain, no rectal bleeding or bloating. Rectal biopsies were taken at colonoscopy and snap frozen in liquid nitrogen. Six micrometer sections of the biopsies were taken and fixed in acetone. Polyclonal antibodies recognising $\gamma\delta$ T cells, CD68 +ve macrophages and *E. coli* were used to stain sections by indirect immunofluorescence (IF). Spectrally distinct fluorophores were used in order to achieve co-localisation. Sections were viewed using an epi-fluorescence microscope and were quantified by counting five randomly selected high power fields.
Results: Samples were collected from 11 CD patients and 5 controls. Co-localisation of *E. coli* with $\gamma\delta$ T cells was seen in 9/11 CD patients but none in controls.$\gamma\delta$/*E. coli*/+ve T cells were observed in both colonic epithelium as well as lamina propria and where present, accounted for ~40%of the total $\gamma\delta$ T cell count.
Conclusions: This is the first study to demonstrate the uptake of *E. coli* in a population of $\gamma\delta$ T cells within the colonic tissues of CD patients but not controls. The results above suggest that the presence of *E. coli* within two immunologically distinct populations of cells may impair the host response and therefore be critical in the resolution of the chronic inflammation seen in Crohn's disease.

1. Wu, Y., *et al.* (2009). Human gamma delta T cells: a lymphoid lineage cell capable of professional phagocytosis. *Journal of immunology* 183(9), 5622–9.

P1098
Regulatory role of the hypothalamic-pituitary-adrenal axis and adrenal glands in experimental colitis

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Purpose/Objective: Inflammatory immune responses may be modulated by the hypothalamic-pituitary-adrenal axis (HPA) through neuroimmunoendocrine interactions and cortisol secretion. However, even in the presence of intact adrenal glands patients may develop chronic diseases such as Inflammatory Bowel Disease (IBD), which may be caused by an imbalance between regulatory and effector responses in the intestinal mucosa. On the other hand, adrenal glands are also involved in stress response, which may predispose to uncontrolled inflammatory diseases. Then, our objective was to investigate the role of the HPA axis and adrenal glands in experimentally induced IBD.
Materials and methods: C57BL/6 mice were subjected to bilateral adrenalectomy and after a 15 day-surgery recovery period the colitis was induced by oral intake of water containing 3% (w/v) Dextran Sulfate Sodium (DSS) for 6 consecutive days. Animals were daily assessed for weight loss and clinical signs of disease. Mice were sacrificed at 6th day of colitis induction and colon samples were collected to assess cytokine production by ELISA and eosinophil peroxidase activity (EPO) by enzymatic assay. Blood samples were also obtained for evaluation of circulating leukocytes.
Results: Our results showed that colitis was more severe in animals subjected to adrenalectomy, which showed greater weight loss, increased disease clinical score and early mortality when compared to colitis group. The absence of adrenal glands was also related to an increase in pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-17 in the gut, along with an augmentation of IL-10, probably in an attempt to compensate for the exacerbated inflammatory response. Moreover, these local alterations were accompanied by reduced EPO in the intestine and diminished circulating eosinophils in the blood, indicating that adrenal produced hormones and neuroimmune interactions may be involved in the maintenance of the peripheral leukocyte pool and control of exacerbated responses in the gut mucosa.
Conclusions: Taken together, our results showed HPA axis and adrenal glands play an important role in the regulation of systemic leukocytes and local inflammatory response in the gut.

P1100
Role of dendritics cells during IBD

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Purpose/Objective: Dendritic cells (DC) participate in the fine control of immune system by promoting effective immunity against invading pathogens and in the same time by preventing excessive inflammation. Even the phenotype and the function of subsets of intestinal DC are well-characterized in mice, few are known in human. Better knowledge concerning human intestinal DC at steady state allows us to understand the role of these cells during IBD.
Materials and methods: A complex panel was set up to characterize and define intestinal human DC by flow cytometry. Because of the difficulty to isolate significant number of intestinal dendritic cells from

controls and IBD patients, an *in vitro* system of intestinal-like DC was develop from monocytes-derived DC (MODC) which could provide sufficient numbers of DC to assess their function.
Results: Our human data indicate that 20% of intestinal DC expresses CD103 while this molecule is not expressed in circulating DC in the blood, suggesting that human CD103+ DC as mice CD103+ DC might be important in maintaining intestinal homeostasis. Moreover gene expression in CD103+ and CD103$^-$ DC from human colon indicates that CD103+ DC express high level of mRNA involved in TGF-β pathway or in enzyme producing retinoic acid (ALDH1A2) compared to CD103- DC. Furthermore CD103+ DC coming from IBD patient's loss the capacity to up regulate TGF-β pathway gens and ALDH1A2.

Besides the culture and the differentiation of CD14+ monocytes from blood of control donor into intestinal-like DC with in presence or in absence of RA or FLT3 ligand or TGF-β indicate that only RA is a potent inducer of CD103 on MODC. Quantitative PCR analysis shows that CD103+ MODC up regulate mRNA related to TGF-β pathway as well as ALDH1A2 compared to CD103- monocytes-derived dendritic cells.
Conclusions: Preliminary data on human intestine suggest that intestinal DC express CD103 and gene related to tolerance. When cultured with RA, MODC expressed CD103 which provide intestinal-like DC phenotype. This CD103+ DC are enriched for genes related to TGF-β activation.

P1101
Th17 related genes and celiac disease susceptibility

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Purpose/Objective: Celiac disease (CD) is an inflammatory intestinal disorder caused by gluten ingestion in genetically predisposed individuals. Th17 immune response has been related to different autoimmune diseases such as Crohn's disease, psoriasis or ankylosing spondylitis. Polymorphisms in genes involved in the Th17 pathway, such as *IL23R*, have been associated with susceptibility to those diseases. We aimed at exploring the role of Th17 cells in CD by studying the association of numerous single nucleotide polymorphisms (SNPs) located in Th17 genes with CD susceptibility.
Materials and methods: We initially studied 735 CD patients and 549 healthy individuals, and we used a replication sample set consisting of 294 CD patients and 475 healthy individuals. All included individuals were Spaniards and Caucasian. We selected 101 SNPs in 15 Th17 genes (*IL23R, RORC, IL6R, IL17A, IL17F, CCR6, IL6, JAK2, TNFSF15, IL23A, IL22, TBX21, SOCS3, IL12RB1* and *IL17RA*) by performing an aggressive tagging using the Haploview program. Genotyping was performed by 'Veracode' technology at the National Genotyping Center (CEGEN) or by Taqman technology in the case of the *IL6*, *IL6R* and *TBX21* genes. Genetic frecuencies were compared between cases and controls using the chi-square test. Interactions between genes were evaluated following four different approaches: logistic regression, random forests (RF), classification and regression trees (CART) and multifactor dimensionality reduction (MDR).
Results: In the case-control study, significant results were not obtained for any SNP with the excepcion of rs4969170 located in the *SOCS3* gene [$P = 0.0018$, OR (95% CI) = 0.59 (0.42−0.84)] and one haplotype conformed by the SNPs located in the *IL23R* locus. However, these results were not replicated in our validation sample set.

No significant interactions between the studied genes were found.
Conclusions: Genetic polymorphisms in Th17-related genes do not seem to be crucial for CD development.

P1102
The Gut Microbiota and Inflammatory Immune Diseases

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Purpose/Objective: The current work will investigate the effect of the microbiota in autoimmune disease in two disease models: inflammatory bowel disease, a local autoimmune disease within the gut, and multiple sclerosis, a systemic autoimmune disease. We aim to identify key bacterial species present in healthy animals and to investigate the presence or absence of these microbes in diseased states. The effect of the microbial composition of the gut on the function of the cells of the immune system in healthy and colitic mice will be assessed through FACS analysis. In addition, the importance of close association of the bacteria to the mucosa to elicit their effect will be studied.
Materials and methods: Denaturing Gel Gradient Electrophoresis (DGGE) has been utilised to expand our knowledge of the microbial composition of the gut. Fluorescence *In Situ* Hybridisation (FISH) was also used to provide information about the location of the bacteria within the gut tissue. A protocol to retrieve the mucosa-associated bacterial population from the gut was developed. The DNA collected from scraping the mouse gut mucosa was run on DGGE gels to visualise the differences between tissues, and between bacteria that are loosely and tightly associated to the mucosa.

Further work will involve a mouse study in which wild-type and IL-10 KO mice are treated with antibiotics. The mice are then treated with bacteria (either a single strain or a cocktail) to assess the effect of the known bacteria on microbiota composition and the host immune system. FACS analysis will investigate the effect on the differentiation of the cells in the immune system. Of the 454 Pyrosequencing and qPCR will identify the exact species present and qPCR enables the quantification of the species.
Results: Thus far, DGGE profiles have been obtained of both faecal and gut mucosa bacterial profiles. Additionally, FISH has been carried out to visualise the location of the bacteria within the gut tissue, while the protocol to retrieve bacteria associated with the gut mucosa is under-going final optimisation.
Conclusions: Our initial work shows a large difference between the bacterial DGGE profiles from faecal samples of wild type mice and IL-10 KO mice. The profiles of loosely and tightly mucosa-associated bacterial communities show a clear difference. FISH results show the technique to retrieve the bacteria from the mucosa was effective.

P1103
The impact of NOD2 3020insC mutation in microbial-driven host innate immunity

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Purpose/Objective: NOD2 is an intracellular pattern recognition receptor of the NOD-like receptor family. Activation of NOD2 by the muramyl dipeptide (MDP) motif of the bacterial peptidoglycan drives NF-κB signaling. The NOD2 *3020insC* polymorphism is a well-established genetic risk factor for Crohn's disease. At present the impact of this mutation on enteropathogen-mediated host innate immune responses is unknown.
Materials and methods: THP-1 cells transduced with WT and *3020insC* NOD2 under both an intermediate and high expression promoter were utilized. PMA-treated cells were stimulated with TLR

(LPS & PGN) and NLR (ieDAP & MDP) ligands and infected with *Campylobacter jejuni*, *Clostridium difficille*, *Enteropathogenic E. coli* and *Salmonella enterica*. Host innate immunity was quantified by gene and protein analysis, flow cytometry and confocal microscopy.
Results: MDP reduced PGN-mediated TNFα levels in cells overexpressing wild-type NOD2 by 40%, indicating that NOD2 is a negative regulator of TLR2-driven pro-inflammatory responses. Enteropathogens led to a marked increase in TNFα, while inhibiting IL-10, during co-infection with macrophages expressing the *3020insC* mutant.
Conclusions: Our observations suggest that *3020insC* NOD2 mutant receptor responds differentially to exogenous stimuli when compared to its WT counterpart. The observed disequilibrium between the pro- and anti-inflammatory cytokine axes may contribute to bacterial-driven IBD pathogenesis in individuals carrying the *3020insC* NOD2 mutation.

P1104
The influence of cell free probiotic supernatant on bacterial macrophage interactions

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Purpose/Objective: Probiotics have been shown to be beneficial on patients suffering from inflammatory bowel diseases such as ulcerative colitis. However, the mode of action is unclear with many papers reporting contrary stimulatory or suppressive effects on immune cell activity.
Materials and methods: This study utilised a gentamicin protection assay (GPA) to assess the influence of probiotics on both the ingestion and digestion phases of phagocytosis by immune cells. The GPA was performed with *E. coli* and a murine macrophage (J774) at a multiplicity of infection of 50:1 in DMEM (Dulbecco's modified Eagle's medium) alone or DMEM supplemented with either 20 μg/ml lipopolysaccharide (LPS), 10% cell-free *Lactobacillus rhamnosus* GG (LGG) probiotic bacterial supernatant or a combination of LPS-LGG.
Results: Studies monitoring bacterial ingestion, demonstrated a significant reduction in bacterial uptake by macrophages when treated with LGG and the LPS-LGG combination ($P < 0.05$). The LPS alone had no significant effect on bacterial ingestion. In studies monitoring bacterial digestion, the LPS and the LPS-LGG combination, brought about significant increase in the digestion rate when compared to control ($P < 0.05$). LGG alone had no significant effect on bacterial digestion.

The data suggest both LGG and LPS modulate immune cells but in a contrary manner. The LGG inhibits bacterial ingestion, but does not influence digestion, whereas LPS does not influence ingestion but enhances bacterial digestion.
Conclusions: By interfering with macrophage ingestion, LGG may suppress the total microbial load associated with macrophages, and, hence, the extent to which pro-inflammatory molecules such as nitric oxide and oxygen free radicals are generated. The suppression of inflammatory promoting signals may be beneficial to the host, since overproduction of these signals may induce host damage. Future work will focus on whether the LGG driven suppression of ingestion is also associated with a reduction of pro-inflammatory molecules and can maintain this suppression in the presence of LPS, a molecule known to stimulate inflammation.

P1105
The TGF-ß activating gut-associated integrin avß8 is upregulated during DC maturation

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Purpose/Objective: Maintenance of homeostasis within the gut requires constitutive immune suppression to prevent inappropriate inflammation, punctuated by effective antigen-specific responses against pathogens. Breakdown of suppression, or failure to deal with infection, can result in inflammatory or allergic disease. Thus, understanding the cellular and molecular mechanisms that regulate intestinal immunity will be important in identifying potential therapies to improve immune-mediated disorders of the gut. We have previously found that high expression of integrin $\alpha v\beta 8$ on lamina propria dendritic cells (DC) results in activation of the key cytokine TGF-β, which induces either Treg or Th17 cells, depending on the immune context. This pathway is critical to gut homeostasis, as shown by CD11c-specific $\alpha v\beta 8$ knockout mice which develop severe colitis. The aim of this current study is to understand how expression of integrin $\alpha v\beta 8$ on DC is controlled, and how this affects downstream immune responses.
Materials and methods: Human monocyte derived DC was treated with intestine-associated molecules and the effect on DC maturation and $\alpha v\beta 8$ expression was measured by flow cytometry.
Results: The TLR ligand LPS upregulated expression of the TGF-β activating integrin $\alpha v\beta 8$ on human DC in a dose-dependent manner. This upregulation appeared to correlate with the activation status of the DC; however, if DC were treated with gut-associated molecules that dampen activation, LPS was still able to cause upregulation of the integrin.
Conclusions: Integrin $\alpha v\beta 8$ upregulation on DCs by danger signals in the gut may contribute to constitutive suppression of non-specific immune responses, or enhancement of Th17 responses depending on the immune milieu. We are currently determining the functional significance of this integrin upregulation in controlling gut immune responses, which may prove to be a potential pathway for modulation of intestinal immunity.

P1106
Tumour necrosis factor alpha in non-inflammatory bowel disease enterocutaneous fistulas prospective study

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Purpose/Objective: The aim of this study is to assess the inflammatory activity, with a particular emphasis on tumour necrosis factor alpha (TNF-α) of non-inflammatory bowel disease enterocutaneous fistula (non-IBD ECF) when compared with IBD ECF and control small bowel terminal ileum tissue. If this study can show the presence of TNF-α in the fistula tract then there would be a potential for a novel therapy for patients with persistent ECF not associated with IBD. This would be an alternative option and benefit an already surgically challenging group of patients associated with a high morbidity and mortality where it is deemed conservative medical management has failed.
Materials and methods: Research ethics approval was granted for this study. Tissue biopsies were obtained from ECF at operation from non-IBD patients and from the terminal ileum in normal colonoscopy control patients. After 24 h incubation, intra cellular staining was performed using monensin to assess the on-going intra cellular production of cytokines. Data was acquired using FACS Canto II.

Further compensation and analysis of list-mode data were carried out subsequently using Win List software.

Results: Results are available on ten non-IBD ECF and four control patients. The student *t*-test for non-paired data was used. Production of TNF- α by dendritic cells from non-IBD ECF tissue was significantly higher than that from control terminal ileum tissue ($P = 0.0008$). All viable cells from non-IBD ECF tissue also produced significantly higher level of TNF- α compared with control tissue ($P = 0.01$). There were no significant differences in the production of TNF alpha, IL17a and IFN-g by T cells in non-IBD ECF compared with cells from control terminal ileum tissue. Ongoing production of IFN-g gamma was not significantly different in all viable cells form non-IBD ECF compared with all viable cells from control terminal ileum tissue. However, the on-going production of IL17a in all viable cells from non-IBD ECF was significantly higher compared with all viable cells from control terminal ileum tissue ($P = 0.04$).

Conclusions: Although this work is still in progress, a trend has been found in the on-going production of cytokines in non-IBD ECF compared with control tissue. Addition of a greater number of samples may show significant differences between the two groups of patients and provide validity to this trend. This data is encouraging and may provide evidence for the potential use of anti-TNF- α agent in the treatment of non-IBD ECF.

P1107
Vagal anti-inflammatory reflex in dextran sodium sulfate (DSS)-induced colitis

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Purpose/Objective: The cholinergic anti-inflammatory pathway (CAIP) is a recently identified endogenous anti-inflammatory mechanism modulating the immune system. We recently established that the CAIP is endogenously activated during subtle inflammation of the small intestine in a model of postoperative ileus (POI). To what extent this neuronal pathtway is also activated during colitis remains however unknown. Therefore, we evaluated vagal activation upon DSS-induced colitis using cFos expression, a marker for neuronal activation, in the dorsal motor nucleus of the vagus nerve (DMV) at different time points of DSS exposure. Moreover, selective vagal denervation of the proximal part of the colon was performed to investigate the anti-inflammatory role of the vagal innervation of the colon.

Materials and methods: C57/Bl6 mice were exposed to DSS in drinking water for 7 consecutive days. Mice were sacrificed at day 1, 3 and 7. The group of mice exposed to 7 days of DSS was divided into 2 experimental groups: vagal denervated (Cx) and sham-operated. Brain was collected for cFos expression and colonic tissue was collected to determine the expression of inflammatory cytokines.

Results: Mice exposed to 7 days of DSS exhibited a significant increase in the disease index activity and a decreased body weight. The inflamed colons showed an increased expression of IL-6, TNFα, and IL1β compared to control mice. Colonic inflammation did not significantly increase cFos expression in the DMV. Denervation of the proximal colon induced an increase of IL6 and IL1β transcript levels in the entire colon. Strikingly, the enhanced colonic inflammation was observed only in the distal part (non-denervated) of the colon.

Conclusions: In the present study, DSS-induced colonic inflammation did not trigger endogenous activation of CAIP as described during intestinal muscularis inflammation in POI. Denervation of the vagus nerve (innervating the proximal part of the colon) enhanced colonic inflammation, confirming the role of the cholinergic anti-inflammatory pathway in mucosal immune homeostasis. Strikingly, removal of this neuronal pathway affects the innate immune response of the distal colon rather than the proximal colon. Our data demonstrated that neuronal circuitry underlying the cholinergic anti-inflammatory mechanism in colitis differs from the CAIP activated during POI.

Poster Session: Malaria

P1108

BTLA expression dampens innate as well as adaptive immunity against experimental malaria

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Purpose/Objective: The activation of T cells during priming but also their later function is modulated by numerous surface receptors. Activation requires signals from the TCR and costimulatory signals, which determine whether antigen recognition leads to full activation or anergy. In contrast coinhibitory receptors expressed by T cells mediate the regulation of immune responses and play a pivotal role in the maintenance of peripheral tolerance. By integrating positive and negative signals excessive activation of T cells is prevented. During malaria T cells express different coinhibitory receptors, which might protect the host from an overreaction of the immune response but might contribute to an ineffective clearance of the pathogen. Recently, BTLA (B and T lymphocyte attenuator, CD272) was described as a novel negative costimulatory receptor. BTLA is predominantly expressed on T and B cells and dampens T cell activation. In this study, we analyzed the function of BTLA during experimental malaria infection.

Materials and methods: To study the function of BTLA we employed a mouse model of blood stage malaria using *P. yoelii* NL. This infection causes a high parasitemia in infected mice that is cleared within 3 weeks. By using BTLA-deficient and HVEM-deficient mice the respective function of these molecules were analysed. Using mixed bone-marrow chimera the function of BTLA on different immune cell populations was further elucidated.

Results: BTLA expression restricts the protective immune response on different levels. BTLA::HVEM interaction regulates the number and function of the responding T cells but in addition BTLA expression also dampens B cells and phagocytic cells. However, in contrast to the manipulation of the CTLA − 4 pathway, where already a transient blockade is accompanied by a massive inflammation, the lower parasitemia of BTLA- and HVEM-deficient mice is not accompanied by any signs of pathology.

Conclusions: In contrast to other negative costimulatory pathways, the HVEM::BTLA pathway dampens the function of the innate as well as of the adaptive immune system during experimental malaria and thus manipulation of this pathway is an attractive target for therapeutic interventions.

P1109

CTLA-4 expression on T effector and T regulatory cells in experimental malaria

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Purpose/Objective: In the course of an infection with *Plasmodium* ssp. the parasites develop via two stages: at first in the liver followed by replication in the blood. In the first phase only few hepatocytes are infected and the antigenic load is low, but in the subsequent blood stage many erythrocytes are infected which leads to a vast amount of antigenic material delivered to the immune system. This induces a strong activation of T cells followed by the production of proinflammatory cytokines that contribute to the severe complication of malaria, the cerebral Malaria (CM). Thus the immune response has to be tightly regulated to achieve on the one hand protection against the parasite but on the other hand prevent immunopathology. CTLA-4 is one of the most effective regulators of T cells and is expressed on Treg and T effector cells. We investigated CTLA-4 expression on T cells in a mouse model for the blood stage of protection against malaria and in a model for CM.

Materials and methods: We infected C57Bl/6 mice and DEREG mice (DEPletion of REGulatory T cells) with *Plasmodium yoelii* or *Plasmodium berghei* ANKA and analysed spleen cells during the development of the disease.

Results: In the course of infection with *P. yoelii* the ratio of Treg to $CD4^+$ T cells declines but the number of CTLA-4 positive T cells increases. These CTLA-4^+ cells produce IFN-g whereas the $Foxp3^+$ cells produce IL-10. After depletion of Treg in DEREG mice with Diphtheria toxin the number of CTLA-4^+ Foxp3- T cells increases rapidly and IFN-g production of $CD4^+$ T cells is also increased whereas TNF-a production is unchanged. However this has no influence on the parasitemia.

When we infected mice with *P. berghei* ANKA we observed a massive induction of CTLA-4 on $CD4^+$ as well as on $CD8^+$ with up to 30% CTLA-4 positive cells. The $CD4^+$ CTLA-4^+ cells are still functional and produce IFN-g but no TNF.

Conclusions: Our data suggest that the rapid induction of CTLA-4 on T effector cells during an infection with Plamodium is a highly dynamic counterregulatory pathway that protects the host from pathology.

P1110

P. berghei sporozoite challenge of vaccinated BALB/c mice lead to induction of humoral immunity and improved $CD8^+$ T cell memory

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Purpose/Objective: Protection against malaria can be achieved by activation of a strong $CD8^+$ T cell response against the *Plasmodium* circumsporozoite protein (CSP). However, most subunit vaccines fail to induce a sufficient memory response. In the present study we analysed the impact of a sporozoite infection after immunization on the development of long-lasting protective immunity.

Materials and methods: BALB/c mice were immunized by a heterologous prime/boost regimen against *P. berghei* CSP. A recombinant *Salmonella typhimurium* strain and a *Bordetella pertussis* adenylate cyclase toxoid fusion molecule were used as vaccine carriers. This immunization induces a strong $CD8^+$ T cell response and complete protection upon sporozoite challenge, which is however, only short lived.

Results: A sporozoite challenge after immunization led to prolonged protective immunity. Repeated challenge infections induced sporozoite specific antibodies that showed protective capacity. On the other hand the CSP-specific $CD8^+$ T cell response was not substantially boosted by sporozoite infections and the magnitude of the $CD8^+$ T cell memory seemed not to correlate with protection. The phenotype of these memory T cells was comparable; however, CSP-specific memory $CD8^+$ T cells of challenged mice displayed stronger cytotoxic activity than

memory T cells of only immunized mice. Sterile protection was abrogated when $CD8^+$ T cells were depleted whereas $CD4^+$ T cells played a minor role.
Conclusions: Based on these data we suggest that the increase in protective immunity observed after immunization and subsequent challenge infection is the sum of different antiparasitic mechanisms including $CD8^+$ and $CD4^+$ T cells as well as neutralizing antibodies, with $CD8^+$ effector-memory T cells playing the major role. Our results indicate that a vaccine which induces a short-lived liver-stage specific immunity that prevents disease within a certain time span would allow the induction of naturally acquired immunity upon repeated sporozoite infections.

P1111
Pathogenesis and therapy of malaria-associated acute respiratory distress syndrome

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Purpose/Objective: Malaria-associated acute respiratory distress syndrome (MA-ARDS) is a lethal complication of malaria. No efficient treatment is available for this complication and its pathogenesis remains poorly understood. We studied the disease mechanisms and evaluated candidate treatments in a new mouse model.
Materials and methods: We established a novel mouse model of MA-ARDS by infection with *Plasmodium berghei NK65* (*PbNK65*). Histology, FACS analysis, RT-PCR, bronchoalveolar lavages, cell depletions and anti-inflammatory treatment with dexamethasone were performed. A new method for the extraction and quantification of hemozoin (malaria pigment) in tissues was optimized.
Results: *PbNK65* infection resulted in leukocyte accumulation, extensive edema and hemorrhage in the lungs. The pulmonary expression of several cytokines and chemokines was increased to a higher level than in mice infected with *P. chabaudi AS*, a parasite strain which does not cause MA-ARDS. $CD8^+$ T lymphocytes were shown to be pathogenic and high doses of dexamethasone (DEX) blocked MA-ARDS through inhibition of lymphocyte proliferation and expression of chemoattractants for monocytes/macrophages, even when given after appearance of the pathology. On tissue sections, we noticed the presence of hemozoin or malaria pigment in the lungs. In view of the important inflammatory potential of this hemoglobin degradation product, we optimized a novel method to quantify hemozoin in tissues and measured the Hz content in different organs of mice infected with parasites of different pathogenicity. Significantly higher amounts of hemozoin were found in the lungs of mice with MA-ARDS. Furthermore, total Hz contents (including liver and spleen levels) were significantly higher in mice infected with *P. berghei NK65* than in mice infected with *P. chabaudi AS*, despite of similar peripheral parasitemia levels. Further investigations to clarify the role of hemozoin in MA-ARDS are currently underway.
Conclusions: Our new mouse model for MA-ARDS has many similarities to human MA-ARDS. Inflammation has a prominent role in the disease, and anti-inflammatory treatment appears highly effective. High amounts of hemozoin were detected in the lungs and may contribute to the pathogenesis.

P1112
Abstract withdrawn.

P1113
The immuno-stimulatory and protective effect of the novel nanoparticle-coated *Plasmodium yoelii* merozoite surface protein (PyMSP-1)

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Purpose/Objective: In malaria DNA vaccine development, for induction of an efficient and protective immunity against parasite, there exists a critical need for additional delivery vehicles. We analyzed the immuno-stimulatory effect of PEI/γ-PGA nanoparticle (NP)-coated PyMSP-1 plasmid and investigated its *in vivo* stimulatory effect on dendritic cells.
Materials and methods: Groups of C57BL/6 mice were immunized either with 100 μg/mouse of nanoparticle-coated plasmid (pVR1020-MSP-1/PEI/γ-PGA), naked (pVR1020-MSP-1) or coated control group (pVR1020/PEI/γ-PGA) using different routes of administration. Mice were prime-immunized at day 0 and subsequently, two boosters given with 3 weeks intervals. Two weeks after the last boost, specific IgG and their subtype's titres measured by ELISA. Cytokine (IL-12 and IFN-γ) levels were measured in the supernatants of antigen stimulated spleen cells and sera from immunized mice using procarta-immunoassays kit. Flow cytometric analysis of various activated DC markers was also carried out.
Results: Protection against *P. yoelii* lethal challenge, specific IgG and its subtypes and INF-γ producing cell number were observed to be significantly higher in the coated-MSP-1 group than the naked group. Also, there were a significantly increased proportion of activated DCs and their elevated CD40 expression in the NP-coated immunized group as compared to naked plasmid. In the coated group, the co-stimulatory molecule CD80 was significantly increased when immunized subcutaneously, while CD86 molecule was increased when immunized intraperitoneally. *In vivo* and ex-vivo production of IL-12 were observed in the sera and the spleen cells stimulated with recombinant MSP-1.
Conclusions: These data indicates that nanoparticle-coated PyMSP-1 DNA vaccine protected mice and induced activated DCs either with CD80 or CD86, and the activated DCs produced IL-12 when stimulated by rMSP-1.

Poster Session: Metabolic Disease and Diabetes

P1115

B cell over-expression of Fc gamma receptor IIb inhibits the development of atherosclerosis

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Purpose/Objective: The development of atherosclerosis is significantly regulated by autoimmune and inflammatory processes involving both innate and adaptive immune systems, and is accelerated in the presence of other autoimmune diseases. The inhibitory FcyRIIb has previously been associated with regulation of atherosclerosis development, but the cells types mediating these effects are unknown. Thus, we investigated the effect of B cell-specific FcyRIIbover-expression on the development of atherosclerosis in mice.
Materials and methods: Atherosclerosis-susceptible, low density lipoprotein receptor $^{-/-}$ (ldlr) mice were irradiated and transplanted with bone marrow from B cell FcyRIIb-transgenic (BTG) mice or non-transgenic littermate controls, then fed a high-fat diet for 6 weeks. Atherosclerosis burden and plaque immune cell content was analysed histologically. Immune cell phenotypes were analysed by flow cytometry and cytokine production by intracellular flow cytometry and ELISA.
Results: Compared to normal chow, a 6 week high-fat diet, known to induce CD4+ T cell activation and increased anti-oxidized lipid antibodies, significantly increased MHC class II levels in splenic and lymph node B cells as well as increased levels of BAFF in serum and spleen, suggesting B cell activation in response to high fat feeding. In ldlr chimeras, there were no differences between control and BTG groups in total cholesterol levels, body weight or total blood cell counts. FcyRIIb overexpression suppressed purified B cell proliferation in response to anti-IgM IgG but not F'ab or LPS, and did not affect splenic B cell MHCII or CD40 expression. The level of atherosclerosis at the aortic root was significantly reduced in ldlr/BTG mice compared to littermate controls. The reduced atherosclerosis in ldlr/BTG mice was associated with reduced CD3+ T cell levels within plaques, reduced *ex-vivo* production of IFN-y by both CD4+ and CD8+ T cells, and reduced levels of CD62L*lo* CD44*hi* CD4+ T cells in the spleen, consistent with the previously proposed role of B cells in promoting T cell activation and atheroprogression.
Conclusions: FcyRIIb expression on B cells leads to reduced atherosclerosis, potentially via suppression of pro-atherogenic T cell activation and cytokine production.

P1118

Galectin-3 deficiency preserves pancreatic islets function in basal conditions and under cytotoxic stimuli

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Purpose/Objective: Galectin-3 (Gal-3) is β-galactoside-binding lectin expressed in variety of tissues and cell types and possesses diverse functions, including promotion of inflammatory response and triggering apoptosis. Since it was established that Gal-3-deficient (Gal-3$^{-/-}$) mice are resistant to streptozotocin-induced diabetes and the precise role of Gal-3 in pancreatic islets activity has not been examined so far, our aim was to explore the influence of Gal-3 absence on insulin secretion and apoptosis of pancreatic islets.
Materials and methods: We exposed mouse pancreatic islets (isolated from Gal-3$^{-/-}$ and WT (C57BL/6) mice by collagenase digestion) to proinflammatory cytokines (IL-1β+IFN-γ+TNF-α, 10 ng/ml each) or left them untreated for 24 or 96 h and analysed their survival and function. After incubation, islet apoptosis was measured by histone-DNA ELISA. In order to assess effect of Gal-3 deficiency on insulin secretion, we performed *in vitro* insulin release assay. Isolated islets were first treated with low glucose (1.67 mM) for 1 h and then with high glucose (16.7 mM) and released insulin was measured by ELISA.
Results: Deficiency of Gal-3 promoted better survival of pancreatic islets when they were cultured in basal conditions for 96 h. In line with that, Gal-3$^{-/-}$ pancreatic islets preserved intact insulin secretion compared to WT islets. What is more, Gal-3 deficiency protected pancreatic islets from cytokine-induced apoptosis. Also, after 24 h-long cytotoxic stimulation, Gal-3$^{-/-}$ pancreatic islets retained normal insulin secretion while WT islets had lower secretion. Similarly, Gal-3$^{-/-}$ islets had better response to glucose-stimulated insulin release in basal as well in inflammatory conditions after 24 h of culture.
Conclusions: This study demonstrates that Gal-3 deficiency protects islets from cytokine-induced apoptosis and preserves their function therefore implicating the role of Gal-3 in pancreatic islets apoptosis during inflammation that occurs in diabetes pathogenesis.

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P1120

Glucose tolerance status and hsCRP in a large population-based survey

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Purpose/Objective: To determine whether high sensitive C-reactive protein (hsCRP) levels differ according to glucose tolerance status.
Materials and methods: Data derived from recently completed population-based survey of diabetes, obesity, hypertension and endocrine disease epidemiology in Turkish adult population (n = 26 499), TURDEP-II.
Results: In female hsCRP was significantly higher than in male (mean ± SEM 3.95 ± 0.05 versus 3.53 ± 0.09 mg/dl, p Univariate analysis of variance corrected for age, gender, urban/rural, region, BMI, waist, sBP, dBP, HDL-c, non-HDL-c, 25(OH)D Vit, PTH, creatinine, TSH and FT4 revealed that hsCRP significantly differ across glucose tolerance status pmellitus (DM) and known DM groups [mean ± SEM (mg/dl)]: 4.13 ± 0.25, P = 0.023; 4.49 ± 0.33, P = 0.006; 5.65 ± 0.35, P.
Conclusions: Based on our study results, mean levels of hsCRP increase from NGT through new DM categories. The fact may confirm that any abnormality of the glucose tolerance (pre-DM and DM) is associated with a low-grade inflammatory process. However, in established DM the inflammatory process may not be as important as in the earlier metabolic derangements.

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P1121

High sensitive C-Reactive protein (hs-CRP) and its correlation with angiographic severity of coronary artery disease: an Indian cohort study

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Purpose/Objective: The association between high sensitive C-reactive protein (hs-CRP) levels and the severity of coronary artery disease (CAD) is highly debated. There are very few studies assessing the role of hs-CRP levels with increasing severity of CAD. The aim of our study was to study the correlation of hs-CRP levels with angiographic clinical vessel score in an Indian population.
Materials and methods: Hundred patients of angiographically proven CAD were studied of which 50 patients were of stable angina (Group I), 50 patients of acute coronary syndrome (Group II) (including 35 patients of unstable angina and 15 patients of MI) from a tertiary health center, New Delhi and a third group comprising of 50 age and sex matched healthy controls were studied over a period of 1 year. The hs-CRP levels were measured by ELISA technique and angiographic clinical vessel scoring was done for all patients.
Results: The mean age of the patients was 49 ± 8.8 years (84% men, 16% women). THe mean hs-CRP levels for stable angina (Group I) (6.04 ± 2.51 mg/l) and acute coronary syndrome (Group II) (8.11 ± 2.84 mg/l) were significantly higher in CAD patients than in controls (1.54 ± 1.27 mg/l, $P < 0.001$). High hs-CRP levels were correlated with higher vessel scores indicating a more severe CAD ($r = 0.735$, $P < 0.001$).
Conclusions: Significant correlations were found between the hs-CRP levels and the angiographic clinical vessel score. High hs-CRP levels have a correlation with the increasing disease severity in CAD patients.

P1122

Human a1-antitrypsin Blocks Heat Shock Protein 70 (HSP70)-Induced Inflammation in Pancreatic Islets

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Purpose/Objective: *Introduction.*

Heat shock protein 70 (HSP70) is an abundant chaperone and an endogenous mediator of cell damage. HSP70 enhances inflammation and promotes antigen presentation and subsequent T cell proliferation via membrane CD91. These activities may take part in islet destruction during autoimmune diabetes and during islet transplant rejection. Indeed, circulating HSP70 has been observed in patients with type-1 diabetes. The anti-inflammatory acute phase protein α1-antitrypsin (AAT) promotes islet survival in a transplantation model and in an autoimmune diabetes model. Its protective mechanism of action in these processes is unknown.

Aim.

Examine whether the protective activity of AAT is related to binding and inactivation of HSP70.
Materials and methods: Peritoneal macrophages, bone marrow derived dendritic cells and pancreatic islets were evaluated for inflammatory responses under stimulation with polymyxin B treated recombinant HSP70 (rHSP70) in the presence or absence of clinical-grade human AAT. Islet function was evaluated by insulin secretion. OT-I spleen cells were incubated with OVA peptide and HSP70 in the presence or absence of AAT. The effect of AAT on IL-1β/IFNγ-induced surface levels of CD91 was evaluated. Binding between HSP70 to AAT was determined by direct ELISA.
Results: In all tested cell cultures, AAT diminished rHSP70-induced inflammatory responses. In HSP70-stimulated islets, IL-1β release was reduced 2.43 ± 2.01-fold, IFNγ 1.73 ± 1.06-fold and IL-6 1.27 ± 1.02-fold. AAT restored rHSP70-mediated disrupted islet insulin secretion. OVA/HSP70 introduced to OT-I spleen cells elevated the number of $CD3^+$ $CD8^+$ T-cells 2.28-fold, compared to cells incubated with OVA alone, and 2.79-fold compared to OT-I spleen cells incubated with HSP70 plus AAT. Under inflammatory conditions, surface CD91 levels were reduced in AAT-treated peritoneal macrophages 2.05 ± 0.33-fold compared to untreated macrophages. Finally, we demonstrate that rHSP70 directly binds to human AAT in a concentration-dependent manner.
Conclusions: Our findings suggest that AAT is a natural regulator of inflammatory responses mediated by HSP70, and a possible target during clinical AAT treatment.

P1123

Interleukin-1 and leptin synergistically enhance matrix metalloproteinase expression in human gingival fibroblasts

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Purpose/Objective: The adipokine leptin is elevated in obesity and type 2 diabetes, conditions which are associated with increased susceptibility to chronic inflammatory diseases such as periodontitis. Loss of tissue integrity in inflammatory diseases is caused by extracellular matrix (ECM) degradation by proteases, such as the matrix metalloproteinases (MMPs). Gingival fibroblasts are critical in regulating tissue homeostasis in the periodontium and synthesise MMPs in response to cytokines but the effect of leptin on these cells is unknown. Our aim was to investigate the effect of leptin in regulating MMP expression in primary human gingival fibroblasts (HGFs).
Materials and methods: HGFs were isolated from gingival tissue obtained during canine exposure surgery. HGFs (between passage 5–9) were stimulated under serum-free conditions with leptin (10 μg/ml), interleukin (IL)-1α (0.05 ng/ml) and oncostatin M (OSM) (5 ng/ml) for 24 h. MMP mRNA expression was assessed by real-time and conventional RT-PCR. HGF proliferation was determined using a MTS-based assay.
Results: Leptin significantly increased the expression of the collagenase MMP-1 in a dose-dependent manner, but did not affect the expression of the gelatinase MMP-2, compared to unstimulated HGFs. MMP-3, a stromelysin known to activate MMP-1, was expressed in both leptin-stimulated and unstimulated HGFs. Interestingly, leptin+IL-1 synergistically increased both MMP-1 and MMP-3 expression in HGF cultures above that observed after IL-1 or leptin stimulation alone. Leptin+IL-1 had no effect on MMP-2 expression. It is unlikely that the synergistic upregulation of MMP expression was due to increased HGF proliferation as leptin+IL-1 stimulation did not increase HGF proliferation above that of cells stimulated with leptin alone. Leptin+OSM did not synergistically upregulate MMP-1, MMP-2 or MMP-3 expression in HGFs.
Conclusions: We demonstrate that HGFs respond to leptin by upregulating MMP-1 expression. The synergy demonstrated between leptin and IL-1 highlights the importance of combinations of cytokines in regulating MMP expression. This pathway may be of mechanistic relevance to inflammatory tissue damage as occurs in periodontitis, and underpin the cross-susceptibility between inflammatory diseases associated with obesity.

P1124

Investigation of possible relations of SDF-1 and CXCR-4 polymorphisms and CD55 and CD59 markers with etiopathogenesis and prognosis in Type 2 diabetes

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Purpose/Objective: Diabetes is a metabolic hyperglycemic disease resulting from impaired insulin production, secretion or insulin resistance. Complement regulators and chemokines are pivotal in pathogenesis. In the context of complications related to T2DM, CD55, CD59 expressions, and SDF-1, CXCR-4 polymorphisms were investigated.
Materials and methods: Seventy-five patients with T2DM and 73 healthy subjects were enrolled in the study. CD55 and CD59 expressions were evaluated with flow cytometry. DNA was isolated from heperinized periferic blood samples. Real-time PCR assay was carried out using LightSnip (rs17881575 Roche-Germany) for SDF-1 and LightSnip (rs2680880 Roche-Germany) for CXCR-4.
Results: CD55 and CD59 expressions in patients with T2DM nephropathy, retinopathy and cardiovascular disease were significantly lower than healthy subjects. SDF-1 genotype and allele distributions between groups were not different. CXCR-4 genotype distribution wasn't different between groups, while a low significance was observed in allel distributions. CXCR-4 T allele was increased in patients, with 1.6-fold risk in terms of disease. Although SDF-1 genotypes in nephropathics did not show any difference, a significant difference was detected for CXCR-4 genotypes. CXCR-4 A allele carriers had decreased nephropathy, while 2-fold high nephropathy frequency was observed in the carriers of CXCR-4 T allele. The nephropathy risk increases 10-fold in CXCR-4 TT genotype carriers. A significant difference was observed in SDF-1 genotypes associated with retinopathy presence. Our results show that all SDF-1 CC genotype carriers have retinopathy, and CC genotype is effective in retinopathy development, however no significance was found for CXCR-4 genotypes. For the presence of cardiovascular disease, a significant difference was observed for SDF-1 genotypes. Increased cardiovascular risk of 5- and 1.9-fold in SDF-1 T and CXCR-4 T allele carriers respectively was observed.
Conclusions: This is the first study investigating together CD55 and CD59 expressions, and SDF-1 and CXCR-4 polymorphisms in T2DM. In conclusion we suggest that CD55 and CD59 have a predictive importance in the process of the disease, and the polymorphism of CXCR-4 gene promoter site (rs2680880) plays a role in the susceptibility to T2DM. Further studies with an increased number of subjects are needed in this field.

P1125

Low frequency of GITR+ cells in *ex vivo* and *in vitro* expanded Tregs from type-1 diabetic patients

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Purpose/Objective: Reported alterations in regulatory T cells (T_{regs}) in patients with several autoimmune disorders led us to a revision of the phenotypical features of type-1 diabetic patients' peripheral $CD4^+$ $CD25^{hi}$ T_{regs} compared to controls.
Materials and methods: A fine cytometric analysis was designed to phenotypically characterise T_{regs} from type-1 diabetic (T1D) patients and controls using a staining panel including FOXP3, CTLA-4, GITR and CD127. PBMCs and sorted T_{regs} were cultured with different stimulus: media alone, soluble OKT3 and anti-CD3/anti-CD28 coated beads. Suppression assays of *in vitro* expanded T_{reg} cells were performed.
Results: The frequency of peripheral T_{reg} cells was similar between T1D patients and controls. However, the yield of sorted T_{regs} was significantly lower in patients than in controls ($P < 0.003$). When comparing T_{reg} phenotype between samples, the only difference concerned GITR. A significant decrease of $GITR^+$ cells within the T_{reg} population ($P < 0.0009$) and to a lesser extent in effector ($P < 0.02$) populations, was observed for T1D compared to controls. Since GITR is involved in costimulation, its expression was analyzed in different conditions of T cell activation. Differences were only observed for T1D T_{regs} versus controls when faced to suboptimal stimulation i.e, soluble anti-CD3 ($P < 0.05$) or medium alone ($P < 0.05$), but not in the presence of anti-CD3/anti-CD28 coated beads. Despite this reduced expression, expanded T1D T_{regs} -mediated suppression was as efficient as that mediated by their control counterparts.
Conclusions: Our results suggested that GITR is a T_{reg}-marker that would be primarily involved in T_{reg} maintenance rather than in its suppressor function.

P1126

Mice deficient for the autoimmune regulator, Aire, display altered T cell responses against 21-hydroxylase, the major autoantigen in autoimmune Addison's disease

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Purpose/Objective: The autoimmune regulator, Aire, is a transcriptional regulator playing a critical role in central tolerance by promoting the display of tissue specific self-antigens. In humans mutations in the gene encoding Aire result in the unique disorder autoimmune polyendocrine syndrome type 1 (APS-1). One of the hallmarks of APS-1 is autoimmune Addison's disease (primary adrenocortical failure) with corresponding immune responses against 21-hydroxylase (21OH), an enzyme crucial for the synthesis of steroid hormones. Mice deficient for Aire also develop spontaneous autoimmunity in several organs, but adrenal autoimmunity or anti-21OH immune responses have not yet been described. We therefore wanted to investigate the influence of Aire on immune responses in mice immunized with 21OH protein.
Materials and methods: C57Bl6 mice deficient for Aire and littermate controls were immunized with 21-hydroxylase (21OH) on day 0 and boosted on day 7. All mice were sacrificed on day 14, and spleens, lymph nodes, adrenals and whole blood were collected. CD4+ and CD8+ T cells were stimulated *in vitro* with recombinant 21OH and a panel of 21OH-derived peptides. Cell culture supernatants were assessed for interferon-γ content by ELISA. T cell mediated cytotoxicity were determined using peptide pulsed or 21OH-transfected EL4 cells as targets.
Results: Broad T cell responses against multiple epitopes of 21OH could be induced in C57Bl6 mice, regardless on the presence or absence of Aire. However, in the absence of Aire, T cell responses were enhanced against certain epitopes. Furthermore, some epitopes were uniquely targeted in Aire deficient mice.
Conclusions: Our findings suggest that T cell responses against 21OH can be readily induced in C57Bl6 mice, and that Aire may modulate these T cell responses.

P1128

Obesity-associated autoantibody production requires AIM for retaining the immune complex on follicular dendritic cells

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Purpose/Objective: Recent evidence has suggested a significant correlation between obesity and autoantibody production, although its mechanism remains unknown. The aim of this study is to address a functional involvement of the apoptosis inhibitor of macrophage (AIM) in this autoimmune process. AIM is a secreted protein produced by macrophages and circulates in blood at around 10 μg/ml in both human and mouse. Interestingly, AIM is associated with pentamers of natural IgM in blood. Natural IgM is polyreactive, recognizing self-antigens, and thus is believed to be associated with autoimmunity. In addition, AIM blood levels increase with progression of obesity in mice when fed with a high-fat diet (HFD). All these observations strongly suggest that AIM/IgM complex might be involved in the development of obesity-associated autoimmunity.

Materials and methods: Following experiments were performed: (1) serum IgM and IgG levels were assessed in $AIM^{+/+}$, $AIM^{-/-}$ and $TLR4^{-/-}$ mice during the progression of obesity, (2) we analyzed autoantibodies in obese $AIM^{+/+}$ and $AIM^{-/-}$ mice using autoantibody array, (3) influence of AIM to the binding of IgM to Fca/mR, an IgM-specific Fc receptor expressed in the splenic follicular dendritic cells (FDCs), was tested. In addition, effect of AIM on the holding of IgM-immune complex (IgM-IC) on the FDCs was analyzed.

Results: In mice fed a HFD, splenic B cells were activated through stimulation of TLR4. This response increased natural IgM production, followed by expansion of IgG autoantibodies. AIM associated with IgM, and inhibited its binding to the Fca/mR such an effect protected IgM-IC from internalization mediated by Fca/mR, and thus retained the ICs on FDCs. This supported IgM-dependent antigen-presentation to B cells leading to plasma cell development. In $AIM^{-/-}$ mice, although elevation of IgM in response to HFD was comparable to $AIM^{+/+}$ mice, the increase of IgG autoantibodies was abrogated.

Conclusions: Our results show that association of AIM with IgM interferes with the internalization of IgM-ICs via Fca/mR, resulting in prolonged retention of the ICs on the surface of FDCs. This contributes to the efficient presentation of autoantigens to germinal center B cells, leading to development of IgG autoantibody producing plasma cells. Thus, suppression of AIM may have therapeutic potential for preventing obesity-associated autoimmune diseases.

P1129

Perivascular adipocytes and signaling through toll-like receptors: role in the pathophysiology of atherosclerosis

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Purpose/Objective: Perivascular adipose tissue has emerged as a critical regulator of vascular function implicated in the pathophysiology of atherosclerosis. Arteries are surrounded by a significant amount of perivascular adipose tissue that is an active endocrine and paracrine source of inflammatory cytokines and adipokines. Moreover, adipocytes express toll-like receptors (TLRs) to respond to lipids and other self and nonself molecules activating proinflammatory pathways. We analyzed TLR/JAK-STAT transduction pathways in adipose tissue from patients with atherosclerosis to unravel the mechanisms implicated in the pathophysiology of atherosclerosis.

Materials and methods: Perivascular and subcutaneous adipose tissues were obtained from control and atherosclerosis patients. Explants of adipose tissue were cultured in presence of TLR ligands and Affymetrix Expression Arrays were performed to analyze changes in gene expression. Western blot was carried out to analyze expression and activation levels of STATs. Immunohistochemistry was used to identify cell types and STATs' activation in adipose tissue. Secretion of adipokines (Adiponectin, Resistin and Leptin) and cytokines (IL-6, IL-10, TNF and MCP-1) in supernatants were quantified by ELISA.

Results: Perivascular adipose tissue from atherosclerosis patients showed a higher expression of genes implicated in immune processes and lower expression of genes implicated in metabolism. Accordingly, perivascular adipose tissue from atherosclerosis patients showed B and T lymphocytes infiltrates.

TLR stimulation of adipose tissue upregulated the expression and activation levels of STATs. While STAT3 was activated at the basal level, STAT1 was activated in tissues previously stimulated with TLR ligands.

Cytokine secretion, especially IL-6, increased in supernatants from explants stimulated with TLR ligands. No significant changes in adipokine secretion were observed after TLR stimulation.

Conclusions: We identified alterations in the TLR/JAK-STAT signaling pathways in adipose tissue that would help to explain the deleterious effects of lipids and other TLR ligands in the pathophysiology of atherosclerosis. The results will be very useful to understand the role played by adipose tissue in atherosclerosis and to design therapeutic approaches to control this inflammatory process.

P1130

Proprotein convertase FURIN is overexpressed in human atherosclerotic plaque leukocytes and associates with vascular stenosis

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Purpose/Objective: Atherosclerosis is a chronic, multifactorial process, where both environmental and genetic factors have critical roles. Proprotein convertase (PCSKs) proteases cleave and convert immature target proteins into a biologically active form. Many PCSK target proteins control various aspects of atherosclerosis thus making also PCSKs as key determinants in atherogenesis. Previously, 4 PCSKs (PCSK5, PCSK9, FURIN, MBTPS1) have been linked to regulation of cholesterol metabolism and plaque inflammation in experimental models.

Materials and methods: We analyzed the expression of PCSK genes in human atherosclerotic plaques and pinpointed the cell types involved. Immunohistochemistry, microarray and Q-PCR were used. To further dissect the role of FURIN in atherosclerosis we genotyped 7 haplotype tagging SNPs (htSNPs) in the FURIN gene region in three independent collections of atherosclerosis patients and controls. We also addressed whether the FURIN expression in the artery wall is genetically regulated by analyzing more than 500 000 SNPs along the genome and reciprocally how the FURIN htSNPs regulate the expression of all the other genes in the artery wall.

Results: FURIN, but not other PCSK genes, was shown to be universally statistically significantly over-expressed in the plaques of different arterial beds. FURIN dysregulation concentrates in the inflammatory plaque cells, such as CD3+ and CD20+ lymphocytes

and CD68+ macrophages. FURIN expression analysis in peripheral blood cells revealed that FURIN was induced in TCR activated CD4+ T cells, as well as upon TLR4 mediated activation of CD14+ myeloid cells.

Analyzing the htSNPs we found that the FURIN SNP rs4932370 associates with the severity of atherosclerosis. SNP genotype − expression correlation analyses revealed that the expression of the ITGBP3 gene is regulated by the FURIN SNP rs12903530. However, after correcting for multiple comparisons no SNPs were found to associate statistically significantly with the FURIN expression in atherosclerotic plaques.

Conclusions: Taken this all together makes FURIN as one of the most interesting candidate genes important in regulation of atherogenesis and the plaque inflammation. However, the FURIN expression is not directly regulated by the polymorphisms and further investigations are needed to decode the factors that contribute to the FURIN regulation in the human atherosclerotic plaques

P1131

Resistin is a risk factor for myocardial infarction and acute coronary syndrome in the general population

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Purpose/Objective: In mice, resistin is an adipokine that plays a role in insulin resistance (IR). On the other hand human resistin is a cytokine whose main source are macrophages and is not relevant in the RI, but is related to atherosclerosis. The few prospective studies have been conducted in patients with acute coronary syndrome (ACS) and resistin has been associated with mortality. It has not been prospectively studied whether resistin modifies the risk of myocardial infarction (AMI) in the general population; that was our goal.

Materials and methods: We measured serum resistin in which so far is the largest general population sample ($n = 6630$). We followed this cohort for 3.5 years (cohort study 'CDC de Canarias'), recording the incident cases of AMI and ACS and calculated the relative risk (RR) of those who occupied the top quintile of resistin (Q5) compared to the rest of the population. We adjusted RR for age, sex and tobacco in Cox models.

Results: Resistin showed higher values in women (5.9 ± 0.5 versus 5.5 ± 0.5 ng/ml; $P < 0.001$). On average Q5 was younger (41 versus 43 years, $P < 0.001$) and had lower prevalence of obesity (26% versus 29%; $P = 0.006$), diabetes (8% versus 11%; $P = 0.013$), hypertension (29% versus 36%; $P < 0.001$) and LDL-cholesterol >160 mg/dl (16% versus 19%; $P = 0.018$), but showed higher smoking prevalence (34% versus 24%; $P < 0.001$). Q5 showed no differences in RI (HOMA2: 0.5 versus 0.51; $P = 0.502$) but had a higher incidence of MI [RR = 2.1 (1.1−3.9); $P = 0.019$] and ACS [RR = 1.7 (1.0−2.7); $P = 0.044$]. After multivariate adjustment, Q5 showed higher incidence of MI [RR = 2.2 (1.2−4.1); $P = 0.012$] and ACS [RR = 1.8 (1.1−2.9); $P = 0.027$].

Conclusions: Elevated resistin acts as a risk factor for AMI and ACS in general population even though Q5 individuals are younger and have lower frequency of known cardiovascular risk factors.

P1132

Serum concentrations and mRNA expression of chemokine CCL17 in patients with type 2 diabetes mellitus and obesity: the influence of caloric restriction and sleeve gastrectomy

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Purpose/Objective: CCL17 (thymus activation-regulated chemokine TARC) is a CC chemokine with chemotactic effects on T_h2 lymphocytes, which are involved in the switch from proinflammatory M1 to antiinflammatory M2 macrophage phenotype in adipose tissue. The aim of our study was to assess the influence of short-term caloric restriction and laparoscopic sleeve gastrectomy (LSG) on serum concentrations and mRNA expression of CCL17 in subcutaneous adipose tissue (SCAT) of patients with obesity and type 2 diabetes mellitus (T2DM).

Materials and methods: Twelve obese females with T2DM undergoing 2 weeks of very-low-calorie diet (VLCD energy content 2500 kJ/day) and 17 non-diabetic women with 3rd grade obesity (BMI > 40 kg/m^2) scheduled for LSG were included into this prospective study. Serum concentrations and mRNA expression of CCL17 together with other investigated parameters were assessed at the beginning and at the end of VLCD and before and 6, 12 and 24 months after LSG.

Results: Both study procedures markedly reduced body weight and improved metabolic parameters including glucose control, insulin resistance and systemic low-grade inflammation. Two weeks of VLCD lead in our obese diabetic patients to a significant increase in serum levels of CCL17 ($P < 0.001$) accompanied by a 7-fold elevation of its mRNA expression in subcutaneous adipose tissue. On the contrary, extremely obese non-diabetic women showed a 30% decrease of systemic CCL17 concentrations at 24 months after LSG, while no significant change could be seen at months 6 and 12. mRNA expression of CCL17 in SCAT exerted a continuous, albeit non-significant, downward trend throughout the whole study period.

Conclusions: Our pilot data suggest the existence of different immunological mechanisms participating in improved metabolic state after short-term and long-term interventions in patients with T2DM and obesity. A more precise definition of the role of CCL17 in these processes might offer novel insights into the mutual link between immunity, inflammation and the pathogenesis of obesity, type 2 diabetes mellitus and their complications.

Poster Session: Neurological and Neuromuscular Diseases

P1134
Ab-proteases and their potential applications in MS diagnostics and treatment

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Purpose/Objective: Here, we aim at elucidating the role of autoantibodies with proteolytic activity (*Ab-proteases*) in MS pathogenesis and discuss their possible applications in MS diagnostics and treatment.
Materials and methods: A comparative mass-spectrometric and kinetic analysis of the activity of Ab-proteases in serum samples from MS patients, their relatives and healthy donor's war performed.
Results: Ab-mediated proteolytic activity showed a marked difference between MS patients and healthy controls and a strong correlation with EDSS scores and thus with different degrees of disability in MS patients.

Besides, 12–18% of the patients' relatives were also found seropositive for MBP-specific Ab-proteases, but those endowed with activity 8–15 times lower (yet significantly higher than that of individuals seropositive for canonical anti-MBP autoAbs). In course of 3–5 years of observation,7 of 12 relatives suspicious for pre-MS showed a gradual increase in the activity of Ab-proteases, and after it reached mid-level, primary clinical and MRI-manifestations could be identified in those relatives. Another significant feature described for Ab-proteases is their sequence specificity. Anti-MBP Ab-proteases that are able to rec-ognize 48–70 and 85–170 amino acid sequences within the MBP molecule are predominantly more typical of MS patients, but not of patients with neurodegenerative diseases other than MS, while Ab-proteases for 43–68 and 146–170 amino acid sequences were found to be occurring only in MS patients. The proteolytic activity of anti-MBP autoantibodies reached its peak at 82–98 amino acid sequence, which had previously been described as having encephalitogenic and immunodominant properties. The experimental *in vitro* application of 82–98 fragment itself (or its mutant form Copaxone), proved able to inhibit MBP-specific at-mediated catalysis.
Conclusions: Ab-proteases appear to be prospective both as potential MS biomarkers and as therapeautic tools. e.g. by changing the sequence specificity of the Ab-mediated proteolysis of the myelin sheath one may reach a reduction in the density of points of the proteolytic effects within the sheath and decrease lesion load, thus minimizing the proteolytic susceptibility of myelin-associated proteins and lowering scales of demyelination. The possibility of designing abzymes for the development of principally new catalysts with no natural counterparts is of great interest too.

P1135
Absence of IGF1R from oligodendrocytes ameliorates EAE

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Purpose/Objective: Signaling of Insulin-like Growth Factor 1 (IGF-1) through the insulin-like growth factor receptor 1 (IGF1R) mediates anti-apoptotic mechanisms in several cell types and regulates differentiation and myelination of oligodendrocyte (ODC) precursor cells (OPCs) during development and following injury. Interestingly, IGF-1 expression is increased in the CNS parenchyma during cuprizone intoxication and surrounding sclerotic lesions in Multiple Sclerosis and in its inflammatory animal model, experimental autoimmune encephalomyelitis (EAE). However, the role of IGF1R signaling in OPCs and ODCs in the context of neuroinflammation remains unclear as published studies show contradictory Results: .
Materials and methods: We used oligodendrocyte-specific deletion of IGF1R to investigate the role of IGF-1 signalling for disease course and recovery in the oligodendrocyte-ablating paradigms EAE and cuprizone intoxication.
Results: The use of a late-acting myelin-specific Cre strain (MOGi-cre) resulted in efficient ablation of the IGF1R gene without any clinical and histological abnormalities developing in the respective animals. We observed, however, upon cuprizone-mediated oligodendrocyte death impaired remyelination. EAE was, surprisingly, ameliorated. We found that microglia was less activated as shown by CD44 and MHC class II surface expression analysis.
Conclusions: Taken together we show that specific deletion of IGF1R from mature oligodendrocytes increases oligodendrocyte susceptibility to death. Yet in EAE absence of IGF1R from oligodendrocytes results in significant amelioration of disease.

P1138
Chronic gastro-intestinal worm infection enhances prion-mediated CNS-pathology and disease incubation time

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Purpose/Objective: Prion (transmissible spongiform encephalopathy) infections are neurodegenerative diseases that affect both humans and animals. Factors which enhance neurodegeneration may be important determinates of the onset of clinical symptoms and disease progression. Pathogen infection is a potential risk factor in the development of other neurodegenerative diseases such as Alzheimer's. Previous studies have shown that the gastro-intestinal worm *Trichuris muris*, which is restricted to the gut lumen, is capable of adversely altering CNS pathology in murine model of stroke through the induction of systemic immune responses. Therefore, the aim of this study was to assess if *T. muris* infection altered CNS pathology and clinical incubation time of ME7 prion disease.
Materials and methods: Groups of mice were infected with ME7 scrapie prions intra-cerebrally (i.c.). At various time-points following ME7 scrapie infection, mice were co-infected with either an acute T_H2-inducing or chronic T_H1-inducing dose of *T. muris* by oral gavage. Mice were culled at the clinical endpoint, after which brains were collected and pathology assessed.
Results: After the onset of TSE pathology, chronic T_H1-inducing *T. muris* infection significantly reduced the time until onset of clinical symptoms and thus the incubation of time of the i.c. scrapie disease. Chronic T_H1-inducing *T. muris* infection prior to the development of TSE pathology enhanced vacuolation in the thalamus and septum but did not alter disease incubation. Infection with an acute T_H2-inducing dose of *T. muris* did not alter either the incubation time or pathology.
Conclusions: Co-infection with T_H1-inducing and not T_H2-inducing doses of *T. muris* is capable of enhancing neurodegeneration in ME7 scrapie-infected mice and accelerating the onset of clinical signs. Determining the basis for this enhancement may help understand how pathogenic infections alter the progression of neurodegenerative diseases and point to novel treatments.

P1140

Donor dependent responsiveness dictates effect of CB2-receptor drugs on human leukocyte migration in response to the chemokine SDF-1

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Purpose/Objective: The aim of this study is to better understand the function of the cannabinoid type 2 receptor (CB2) expressed by certain human immune cells circulating in blood. Various rodent experimental data suggests that CB2 drugs (CB2 receptor selective agonists) have anti-inflammatory properties in models of stroke and multiple sclerosis. The CB2 drugs appear to improve the neurological deficits, by suppressing the detrimental neuroinflammation in these debilitating diseases (often fatal). A proposed mechanism of action is blockade of leukocyte infiltration into the damage brain.
Materials and methods: We assessed whether a variety of cannabinoid ligands had any cytotoxic properties towards human peripheral blood mononuclear cells (PBMC) from blood of healthy volunteers. In addition, we assessed whether cannabinoid ligands had any pro-chemotactic activity as previously suggested by other authors. Furthermore, we have assessed the ability of selective CB2 agonists to abrogate human leukocyte chemotaxis towards well defined pro-inflammatory chemokines, such as SDF-1. Migration assays were performed using 96-well plate HTS Transwell assay coupled with ATPlite measurement of migrated cells.
Results: The cannabinoid drugs we tested were not cytotoxic towards human leukocytes ($n = 6$ donors). Nor did we observe any basal chemotactic or chemokinetic activity of the cannabinoid drugs ($n = 4$ donors). However, several CB2 agonists, (namely HU308 and JWH015), significantly suppressed the chemotactic responses of human leukocytes *from some donors* towards SDF-1 ($n = 6$). Interestingly, this response was donor-dependent as the CB2 agonists had no effect at all or potentiated the SDF-1 mediated migratory response ($n = 3$).
Conclusions: This donor heterogeneity is clinically very relevant for the application of these drugs in human for neurological diseases or other inflammatory conditions where CB2 receptors are a potential target. The variability clealry demands more detailed studies to understand the donor variability and target cells.

P1141

Elevated IL-10 in Dark Agouti rats suffering from experimental autoimmune encephalomyelitis

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Purpose/Objective: Experimental autoimmune encephalomyelitis (EAE) is an animal model of neuroinflamatory and demyelinating disease multiple sclerosis. EAE in Dark Agouti (DA) rats is characterized by strong inflammation with intense infiltration of immune cells into the central nervous system (CNS) leading to neurological impairments. On the contrary, EAE in Albino Oxford (AO) rats is mild, often asymptomatic, while infiltration of immune cells into spinal cord (SC) is limited, yet present. Cytokines produced by immune cells in lymphoid tissues and within the CNS largely contribute to EAE pathogenesis. So, objective of this work was comparison of various cytokine gene expressions in SC of DA and AO rats.
Materials and methods: Rats were immunized with myelin basic protein (MBP) or SC homogenate and complete Freund's adjuvant. SC were isolated from rats at different stages of the disease, and homogenates and SC immune cells (SCIC) were assessed for cytokine gene expression by real-time RT-PCR and protein production by ELISA assay. Cells were isolated from lymph nodes draining the injection site (DLNC) of the animals at the induction phase of EAE and from cervical lymph nodes (CLNC) of unimmunized rats. Also, $CD4^+$ cells were obtained from DLNC by magnetic beads separation.
Results: We found higher expression of pro-inflammatory cytokines IL-17 and IFN-gamma, but also of anti-inflammatory IL-10 at the peak of EAE in SC homogenates and SCIC in DA rats comparing to AO rats. On the contrary, lower expression of anti-inflammatory TGF-beta was detected in DA rats. IL-4 gene expression was extremely low in both strains. Increased gene expression of IL-10 was accompanied with greater IL-10 production in SCIC. Higher IL-10 expression and generation were also recorded in DLNC and $CD4^+$ DLNC, but not in mitogen-stimulated CLNC of DA rats in comparison to AO rats.
Conclusions: Although IL-10 is an anti-inflammatory cytokine with a prominent role in immunoregulation and its role in the course of EAE is thought to be protective, our results showing its higher expression and production in DA rats at the peak of disease imply that IL-10 might not have such a unilateral role in EAE pathogenesis.

P1142

Expression of IL-22 in the central nervous system during development of experimental autoimmune encephalomyelitis (EAE)

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Purpose/Objective: Interleukin (IL)-22 is a member of the IL-10 family, which are important regulators of the inflammatory response. Interestingly, IL-22 is also produced by Th17 cells, which play a key immunopathogenic role in many autoimmune diseases. However, the function of IL-22 in the development of central nervous system (CNS) inflammation is less clear. IL-22 deficient mice are fully susceptible to the development of experimental autoimmune encephalomyelitis (EAE), while IL-22 receptor (IL-22R) is expressed on blood-brain barrier (BBB) endothelial cells and IL-22 disrupts BBB tight junctions *in vitro* and *in vivo*. Therefore, the aim of this study is to examine the expression of IL-22 in the CNS tissues and to investigate whether the expression level correlates with the development of EAE.
Materials and methods: EAE was induced by immunising mice with MOG_{35-55} peptide emulsified in Complete Freunds Adjuvant (CFA), together with intraperitoneal injection of pertussis toxin (PTX). Naive mice or mice immunised with PBS in CFA together with PTX were used as controls. EAE clinical score and mouse weight were examined and recorded daily. At day 9, 17 and 28 post-immunisation, mice were sacrificed and spleen and spinal cord tissues harvested. Single cell suspension of spleen cells were cultured with or without MOG_{35-55} and supernatant were collected for cytokine detection using ELISA. Spinal cord tissues were visualised by histological and immunohistochemical staining.
Results: Our data demonstrate that MOG_{35-55} immunised mice developed EAE around day 9 and reached peak at day 15 while PBS immunised mice remained unaffected. ELISA of the spleen cell cultures show that cells from EAE mice produced more antigen-specific IL-17, IL-22 and IFN-γ at day 9 and day 17. Furthermore, immunohistological staining data show that whilst IL-22 was expressed by naïve/PBS spinal cord tissues, expression was highly up-regulated in the spinal cord of EAE mice.
Conclusions: Our data therefore suggest that IL-22 may play a pathogenic role in autoimmune disease in the CNS, possibly through divergent roles in both the peripheral immune and CNS systems.

P1143

FoxP3+ T-regulatory cells are reduced in peripheral blood in ischemic stroke patients

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Purpose/Objective: Stroke is a leading cause of disability and death. Recently it became evident that cerebral ischemia leads to a defect of the innate and the adaptive immune response which is associated with an increased risk of secondary infections. To date, only scarce information is available on the underlying immunopathology. T-regulatory cells (Treg) are an important inhibitor of immune responses. In experimental stroke conflicting data have been reported regarding the survivial and importance of Treg. This study aimed to quantify and phenotype Treg in the peripheral blood of stroke patients and to assess their function.

Materials and methods: Stroke patients ($n = 38$) and age matched non-stroke controls ($n = 15$) were recruited. Blood samples of patients were obtained on stroke unit admission and on days 1, 3, 5 and 7 thereafter. Treg were phenotyped by staining $CD4^+$ $CD49d^-$ $FoxP3^+$ cells using FACS analysis. Additionally we determined CD39 and CD45RA expression to identify active and naïve Treg.

In a functional assay the suppressive capacity of $CD4^+$ $CD25^+$ $CD127^{dim/-}$ Treg on T-effector (Teff) was evaluated by measuring the expression of CD69 and CD154 on anti-CD3/anti-CD28 stimulated Teff.

Results: In stroke patients the percentage of $CD4^+$ T-cells expressing Treg markers was reduced on day (d) 0 ($P < 0.01$), d1 ($P < 0.01$), d3 ($P < 0.05$), and d5 ($P < 0.01$) compared to healthy controls. This effect was also seen in $CD39^+$ Treg. Functional testing revealed an impaired inhibition of CD154 expression ($P < 0.01$) in stroke derived samples but no difference in CD69 expression.

Conclusions: Our data show that in peripheral blood of stroke patients $CD4^+$ T-cells contain less Treg than in controls. In addition, in stroke patients the suppressive efficacy of Treg appears partly impaired. This could either be due to impaired Treg function or due to altered Teff function. We conclude that stroke induced immune suppression is unlikely to be caused by a selective survival and extensive suppressive activity of Treg. Since we have only access to peripheral blood, we cannot exclude that Treg have migrated to other compartments.

P1144

High levels of free kappa light chains incerebrospinal fluid predict conversion to multiple sclerosis

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Purpose/Objective: A clinically isolated syndrome (CIS) may be the initial presentation of multiple sclerosis (MS). However, some CIS never develop MS. The identification of patients at risk of MS conversion is crucial as early treatment may improve their outcome. Free kappa chains (FKC) are increased in cerebrospinal fluid (CSF) of MS patients. We studied the accuracy of CSF FKC level measurement, using a new nephelometric test, to predict conversion of CIS patients to MS.

Materials and methods: We quantified this protein in CSF from 25 patients with non-inflammatory neurological diseases (NIND) and 78 consecutive CIS patients. We assessed whether high CSF FKC levels associate with CIS conversion to clinically definite MS, defined as the onset of new relapses during follow-up.

Results: A cut-off value of 0.53 mg/l (mean+2SD of NIND group CSF FKC values) was calculated. CIS patients with CSF FKC above this value showed earlier conversion to MS in univariate and multivariate Cox analysis (HR = 6.41; 95% CI = 1.88−21.78, $P = 0.003$).

Conclusions: High CSF FKC levels accurately predict CIS patient conversion to MS.

P1146

High serum LPS values associate with lower disability in multiple sclerosis

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Purpose/Objective: High LPS values may induce endotoxin tolerance in monocytes, which is characterized by a decreased production of cytokines in response to proinflammatory stimuli. We aimed to explore this phenomenon in multiple sclerosis (MS) and to investigate if increased LPS values associate with any clinical or immunological variables in the disease.

Materials and methods: The study included 55 with MS patients and for comparison, 11 patientswhith other inflammatory neurological diseases (OIND), 13 patients with non inflammatory neurological diseases (NIND) and 10 healthy individuals (HC). No differences were found in age or gender between the four groups. We also monitored in MS group disease duration, time to a relapse and disability measured by the EDSS score. Serum samples were stored at −80°C until assayed. LPS was measured under sterile conditions by the endopoint chromogenic Limulus Amebocyte Lysate test (Lonza). Serum levels of activin A were measured by ELISA (R&D). Peripheral blood T and B cell subsets were studiedby by flow cytometry on a FACS-Canto II instrument. Immunoglobulin and albumin indexes were assessed by nephelometry on a Beckmann nephelometer.

Results: We did not find differences in the LPS levels between MS patients and the other patient groups. We classified MS patients in two groups according to serum LPS values and did not find differences between both groups in the percentages of Tor B cell subsets, immunoglobulin indexes or in activin A concentration. However, we found a striking difference in patient disability. Those with high LPS values showed a significantly lower EDSS score ($P = 0.004$).

Conclusions: This data suggest that endotoxin-tolerance induced by high LPS serum values may contribute to regulate the abnormal inflammatory response taking place in MS and ameliorate disease course.

P1147

IL-33 protects mice from experimental autoimmune uveoretinitis

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Purpose/Objective: IL-33 is a recently discovered IL-1 cytokine family member. Previously, we have reported that IL-33 is an important modulator of the immune system and is associated with several immune-mediated disorders. The aim of this study is to evaluate the role of IL-33 cytokine in the development of experimental autoimmune uveoretinitis (EAU).

Materials and methods: The expression of IL-33 and its receptor ST2 on retinal pigment epithelial (RPE) cell line was examined by immunohistochemical staining. Next the severity of IRBP peptide induced-EAU was assessed in C57BL/6 mice treated with recombinant IL-33 or PBS. Cytokine secretion and production by the draining lymph nodes (DLNs) or spleen cells were measured at day 26 after immunization.
Results: We demonstrate that RPE cells expressed high levels of both IL-33 and ST2. Administration of IL-33 cytokine to EAU mice led to reduced disease severity. In line with the reduced inflammation in the retina of IL-33 treated mice, the percentage of IFN-γ+ or IL-17+ cells in the DLNs and spleen was markedly lower, while IL-5+ or IL-4+ cell percentage was increased. Furthermore, antigen specific production of IFN-γ, IL-17 and IL-6 by the DLN cells from IL-33 treated mice was also significantly reduced.
Conclusions: Our results suggest that IL-33 may play a protective role in the development of EAU possibly via its known role in promoting the function of alternatively activated macrophages.

P1148

Is Parkinson's disease the result of autoimmunity arising from Influenza A infection of the brain?

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Purpose/Objective: The mechanism of dopaminergic neuronal cell death remains a mystery in Parkinson's disease. Compelling epidemiological evidence links Parkinson's disease with Influenza A infection, with 5 million people developing the Parkinson's associated disease Encephalitis lethargica following the 1918 influenza pandemic. However, as Influenza induces an acute infection, where as Parkinson's disease is a chronic condition, and in the majority of cases the virus is absent from the lesion, this suggests that the virus has an indirect mode of action. An autoimmune reaction may thus be the effector mechanism linking the infection to neurological disease. Using a murine model we set out to investigate the relationship between influenza A and Parkinson's disease, and in particular, investigate the hypothesis that autoimmunity arising from infection results in dopaminergic neuronal death in the substantia nigra.
Materials and methods: A murine model was established with intranasal delivery of the neurotropic H1N1 A/WSN/33 Influenza strain. Following infection, brains were harvested and examined for the presence of the virus, T cell infiltrate and dopaminergic neuronal loss by immunohistochemistry and flow cytometry. Murine behaviour was also examined for Parkinsonian symptoms. A potential autoantigen, alpha synuclein; a protein central to the pathology in Parkinson's disease, was also examined. Mice were primed against alpha synuclein in CFA and T cell behaviour examined.
Results: Preliminary data using our murine model has shown that the Influenza virus was detected in the midbrain as late as 21 days post infection. T cell subsets were also detected in the brain following infection. In addition to this, we were able to generate alpha synuclein reactive T cells, and these cells were able to traffic to the brain.
Conclusions: Identifying autoimmune mediated dopaminergic neuronal loss would radically change therapeutic approaches and may thus provide new targets to prevent the disease or preserve the quality of life of the patients. We thus need to further examine the aberrant immune response in Parkinson's disease.

P1149

Is there a functional role for KCNMA1 in the multiple sclerosis?

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Purpose/Objective: A more detailed insight into disease mechanisms of multiple sclerosis (MS) is crucial for the development of new and more effective therapies. MS is a chronic inflammatory autoimmune disease of the central nervous system. The aim of this study is to identify novel disease associated proteins that are functionally involved in the MS brain pathology.
Materials and methods: In a previous proteomics study, brainstem proteins were obtained from Lewis rats with MBP induced acute experimental autoimmune encephalomyelitis (EAE), a well characterized disease model of MS. Samples were collected at different time points: just before onset of symptoms, at the top of the disease and following recovery. To analyze changes in the brainstem proteome during the disease course, a quantitative proteomics study was performed using two-dimensional difference in-gel electrophoresis (2D-DIGE) followed by mass spectrometry.
Results: We identified 75 proteins with a significant abundance difference between the different disease stages. Regulated proteins were mapped to existing biological networks by Ingenuity Pathway Analysis (IPA). Post-synaptic density protein 95 (DLG4), a key player in neuronal signalling and calcium-activated potassium channel alpha 1 (KCNMA1), involved in neurotransmitter release, are 2 putative regulators connecting 64% of the proteins identified. The involvement of KCNMA1 in macrophage functionality was studied *in vitro* by using a specific functional blocker for KCNMA1, paxillin. We show that blocking of KCNMA1 altered myelin phagocytosis and proinflammatory cytokine release, disease mechanisms which are highly involved in EAE and MS pathology. We are currently investigating possible influences of this blocker on functionality of other disease relevant cells and processes using *in vitro* and *in vivo* models.
Conclusions: This study will elucidate to what extent modulation via this ion channel affects disease processes in the context of EAE/MS.

P1150

Mast cells protect from post-traumatic spinal cord inflammation in mice by degrading inflammation-associated cytokines via mouse mast cell protease 4

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Purpose/Objective: It becomes increasingly clear that mast cells (MCs) are not only key players in allergic diseases (e.g. asthma), but seem to play a complex role in neuroinflammatory diseases such as multiple sclerosis and stroke. However, their role during and after mechanical CNS trauma is not clear. In the present study, we have investigated the effects of MC-deficiency on the histological and clinical outcome after spinal cord injury (SCI) in mice.
Materials and methods: MC-deficient W-sash c-kit mutant knockout mice ($kit^{W\text{-}sh/W\text{-}sh}$), mMCP-4 deficient ($mMCP4^{-/-}$) mice and control mice underwent spinal cord hemisection at thoracic level T8 resulting in a complete transection of the dorsomedial and ventral corticospinal tract. Functional recovery in SCI mice was tested with the Basso Mouse Scale. Spinal cord sections were analyzed by immunofluorescence. RT-PCR and Western blotting were used to analyze cytokine/chemokine mRNA and protein levels. In degradation assays murine recombinant

IL-1β, IL-4, IL-6, IL-10, IL-13, TNF-α and MCP-1 were incubated with supernatant from BMCMC derived from either C57BL/6 or from mMCP4$^{-/-}$ mice. Cleaved fragments were identified using tris-tricine SDS-PAGE and analyzed by intensity analysis.
Results: We show that MC-deficient *kit*$^{W\text{-}sh/W\text{-}sh}$ mice display significantly increased astrogliosis and T cell infiltration as well as significantly reduced clinical outcome after SCI compared to wildtype mice. In addition, MC-deficient mice show significantly increased levels of MCP-1, TNF-α, IL-10 and IL-13 protein levels in the spinal cord after SCI. Mice deficient in mMCP-4, a MC-specific chymase, also showed increased MCP-1, IL-6 and IL-13 protein levels in spinal cord samples and a decreased functional outcome after SCI. A degradation assay using supernatant from MCs derived from either mMCP4$^{-/-}$ or wildtype mice revealed that mMCP-4 cleaves MCP-1, IL-6 and IL-13, suggesting a protective role for MC proteases in neuroinflammation. These data indicate that MCs may be protective after SCI and that they may reduce CNS inflammation by degrading inflammation-associated cytokines via the mast cell-specific chymase mMCP-4.
Conclusions: In summary, our results suggest a new and complex mechanism how MCs and their proteases may protect the CNS from exacerbated and/or chronic inflammation after damage.

P1151
Peripheral blood CD16+56$^-$ cells may contribute to the early diagnosis of multiple sclerosis

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Purpose/Objective: Alterations in peripheral blood natural killer (NK) cell subset numbers and functions have been linked to multiple sclerosis (MS). NK cells are classified in two major subsets, CD56 dim and CD56 bright, which differ in their functional and homing properties. In early HIV infection, a CD16+ CD56$^-$ NK cells subset are expanded are correlated with a higher plasma viral load set point, suggesting its utility as a predictive marker for disease progression. The aim of this study was to investigate this recently described NK subset in MS and in patients with other neurological diseases.
Materials and methods: We studied 63 patients with relapsing remitting MS, 16 with other inflammatory neurological diseases of the central nervous system (OIND) and 17 patients with non-inflammatory neurological diseases (NIND). Peripheral blood cells were labelled with conjugated monoclonal antibodies and analyzed on a standard FACSCanto-II cytometer (BD). Results were analyzed with the Mann Whitney U-test for comparison between groups.
Results: NK bright and the NK dim cells subsets did not change in MS patients when compared to the other groups. In addition, we did not find differences in CD56$^+$ CD3$^-$ or CD56+ CD3+ NK cells subsets between the three groups of patients. However, CD16$^+$ CD56$^-$ cells were clearly increased in OIND patients when compared to MS ($P = 0.01$) and NIND ($P = 0.0084$) patients.
Conclusions: These results may be of clinical relevance in the early differential diagnosis of MS.

P1152
Protein profiling of the cerebrospinal fluid in diagnostics, prognosis and treatment management of multiple sclerosis

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Purpose/Objective: When dealing with diseases of the central nervous system (CNS), the cerebrospinal fluid (CSF) seems to be the most appropriate body fluid for early and accurate diagnosing. Specifity of any changes found in the protein profile of the CSF is higher, and there is higher possibility for detection of low-abundance proteins. With the CSF it is also possible to skip separation by means of electrophoresis, chromatography etc.
Materials and methods: To access possible diagnostic and prognostic criteria for MS, protein profiling of the CSF was used, using patients with inflammatory and non-inflammatory diseases of the CNS as controls. Protein profiling of the CSF was performed by means of MALDI-TOF mass-spectrometry.
Results: Biomarkers of multiple sclerosis (MS) are devided into 2 groups: markers of immune inflammation and markers of neurodegenerative processes.

Markers of immune inflammation include oligoclonal IgG bands, light Ig chains, chromogranin A, clusrerin, CC3.

Markers specific to autoimmune processes taking place in MS areautoantibodies to myelin basic protein (anti-MBP) and to myelin olygodendrocyte protein (anti-MOG). Antiganglioside antibodies (anti-GM) are more of prognostic value, as there is a correlation between anti-GM antibody type and clinical type of the disease (primary progressive, back-and-remitting, and secondary progressive). Markers of neurodegenereation are components of structures altered during the clinical attack of MS. The best studied marker is the main target of autoimmune reactions in MS myelin basic protein (MBP). High levels of MBP have been shown to correlate with upcoming relapses of MS, as well as with the disease attaining the progressive clinical type. Another prognostic marker is acidic calcium-binding protein a component of axons damaged during the course of the disease. Another important task is predicting transformation of clinically isolated syndrome (CIS) into manifest MS. Some protein markers that have shown diagnostic value are *14-3-3 protein and chemokin CXCL13.*.
Conclusions: Proteomics of liquor gives a possibility of diagnosing the disease and planning the protocols of treatment conveniently and fast, without excessive time and resource consumation. Hovewer, there is a number of methodological problems. One of these is the fact that it is impossible to form control groups of healthy individuals. Another reason is lack of standarts for sample collecting and storing, and lack of international bases including protein profiles of the CSF. Overcoming this obstacle is obligatory for further progress in this field of predictive medicine.

P1153
Regulation of class II MHC antigen expression in microglial cells

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Purpose/Objective: Deregulation of microglial activation mechanisms plays a critical role in the development of neurodegenerative conditions like ageing, Alzheimer's disease, Parkinson's disease, Huntington's disease and schizophrenia. Studies have shown that the

neurodegenerative role of microglia has been highly correlated with the expression of MHC-II molecules. The aim of the present study was to define the membrane and intracellular expression of H-2A as well as the intracellular expression of H-2M, H-2O and CD74 in different activation states of microglial cells, using the BV-2 cell line.
Materials and methods: Flow cytometry and confocal microscopy analysis were used to define the expression of H2-A, -O, and -M molecules in BV-2 cells. Isolated mRNAs were submitted to RT-PCR experiments using *CD74* specific primers. LPS, IFN-γ and IL-4 were used to explore microglial activation mechanisms. Secretion of H2-A molecules in different activation states was evaluated by ELISA experiments.
Results: BV-2 cells expressed all necessary components for post-translational regulation of MHC class II molecules and their transport to the cell surface. Regarding microglial activation mechanisms, LPS did not affect MHC-II expression but induced TNF-a secretion while promoting the cytophagic properties of microglia. The proinflammatory cytokine IFN-γ reduced of MHC-II expression but increased secretion of H2-A molecules as tested by ELISA experiments. IL-4 activation reduced membrane and intracellular H2-A while inducing H2-O expression.
Conclusions: Stimulation of BV-2 cells with LPS, IFN-γ and IL-4 demonstrated that different activation stimuli could lead to different activation pathways of microglial cells promoting a variety of events ranging from neuroprotection to neurodegeneration. Studying the regulatory pathways of microglial MHC II expression could delineate mechanisms of stimulation versus suppression, surface expression versus secretion of MHC-II molecules during pathological conditions in neurodegenerative diseases and dictate new strategies of therapeutic approaches.

P1154
The acute-phase protein Hemopexin regulates Th17 cells and experimental allergic encephalomyelitis development

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Purpose/Objective: Hemopexin (Hx) is an acute phase protein synthesized by hepatocytes in response to the pro-inflammatory cytokines IL-6, IL-1β and TNF-α. Hx is the plasma protein with the highest binding affinity to heme and controls heme-iron availability in peripheral cells and also in T lymphocytes, where it modulates their responsiveness to IFNγ. Recent data have questioned about an anti-inflammatory role of Hx, a role that seems heme-binding independent. The aim of this study was to investigate the role of Hx in the development of a T-cell mediated autoimmune response.
Materials and methods: Experimental autoimmune encephalomyelitis (EAE), the animal model of MS, was induced in WT and Hx knockout ($Hx^{-/-}$) mice. The development of the disease, the levels of Hx, the activation of Th1 and Th17cells and the production of inflammatory cytokines by macrophages were evaluated.
Results: During EAE Hx level increased and remained high. $Hx^{-/-}$ mice developed a clinically earlier and more severe and pathologically exacerbated expression of EAE compared to WT controls. $Hx^{-/-}$ mice displayed a higher amount of CD4+ infiltrating T cells and a more severe demyelization in the CNS compared to WT. The severe EAE developed by $Hx^{-/-}$ mice could be ascribed to the higher amount of Th17 cells infiltrating the CNS and circulating in the periphery of $Hx^{-/-}$ mice compared to WT mice. *In vitro*, T cells from $Hx^{-/-}$ mice polarized more efficiently towards IL-17 producing cells compared to WT lymphocytes. Moreover, the lack of Hx was associated with enhanced production of inflammatory cytokines in the antigen presenting cells, in particular IL-6 and IL-23, the major Th17 differentiating factors.
Conclusions: Over all our observations indicate that Hx could influence the generation of Th17 response during EAE and account for the anti-inflammatory role of Hx in T cell-mediated autoimmune disease.

P1155
The different role of NKT and NK cells functional activity in demyelinating polyneuropathies

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Purpose/Objective: Acquired autoimmune demyelinating polyneuropathies include Guillain-Barr' Syndrome (GBS) and Chronic Inflammatory Demyelinating Polyneuropathy (CIDP), which differ in the etiology (GBS usually develops as a complication of infectious processes, immunopathogenesis, course duration, methods of treatment and therefore call for differential diagnosis.
Materials and methods: Were observed 68 patients with demyelinating polyneuropathiesby flow cytometry who were divided into two groups: 42 GBS patients and 26 with CIDP. Expression of cell-surface markers such as CD3+/CD56+/CD4+, CD3+/CD56+/CD8+, CD3+/CD56+/DN, CD3−/CD16-/CD56+, CD16+/CD56+/CD158a, h+, CD16+/CD56+/CD94+, CD16+/CD56+/NKG2D+, CD16+/CD56+/NKp46+ were assessed by multiparametric FACS.
Results: It was found that in GBS unlike in the CIDP the fall of NKT number was not present but reliably reduced the NK cell number. In all patients D4+ NKT increased 10 and more times, subpopulation CD8+ NKT significantly decreased. The changes in NK cell in GBS patients were accompanied by a decline in their expression of KIR (D158a, h), NKG2D, NKp46. The fall of natural cytotoxicity of receptor content (NKp46) in 1.8-fold on NK membrane is observed only in GBS while negative shift in receptor expression of antibody dependent cytotoxicity is observed in all manifestations ofdemyelinating polyneuropathies.
Conclusions: It is expected that the observed immunopathogenetic features with NKT and NK cells participation can help in differentiation of GBS from CIDP.

P1156
The pro-inflammatory cytokines IL-1ß and TNFa both have deleterious effects on human astrocytes

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Purpose/Objective: Astrocytes play important roles within the human central nervous system through trophic support of neurons and are inextricably involved in the brain's immune status. Astrocytes are part of the blood-brain barrier vasculature, which maintains cellular and soluble traffic into this immune specialised tissue. Any event (immune response, infections, and drug treatments) that is detrimental to astrocyte survival or function will have a negative effect neurologically. Our aim was to investigate acute and long-term influence of neuro-inflammatory events on astrocyte survival and fate.
Materials and methods: We used xCELLigence technology to track astrocyte responses to various inflammatory mediators, in conjunction

with classical assays including cell counts and caspase-3 expression as markers of cell loss/death.xCELLigence is a real-time biosensor technology, which directly measures the level of adhesion of living cells. Theoretically, any treatment that significantly or acutely affects adhesion, (e.g. changes cell morphology, cell proliferation or cell death), can be investigated using xCELLigence, with appropriate validation of the responses. Specific cellular responses produce signature changes in growth curve dynamics and we reveal and explain several of these. All responses were verified and corroborated using conventional assays.

Results: We show that the pro-inflammatory cytokines IL-1β and TNFα are both cytotoxic towards astrocytes. IL-1β is more potent than TNFα, with cytotoxic effects induced in the high fempto-molar range (>250 fM), whereas TNFα induced astrocyte death occurred in the pico-molar range (>250 pM). xCELLigence technology elegantly revealed the differential responsiveness of astrocytes to IL-1β and TNFα, and other cytokines including IFNγ, CXCL8 and IP10, which had no influence on astrocyte viability but have important roles in neuroinflammation.

Conclusions: Certain pro-inflammatory environments clearly have a detrimental effect on these very important brain immune cells. These findings have important implications for understanding the adverse events occurring during neuroinflammation that exacerbate or perpetuate the primary insult. Similar events may occur in brain diseases such as stroke or MS, where vascular inflammation and damage are hallmarks of the disease.

P1157

The role of iron in inflammatory demyelinating diseases: development of experimental *in vitro* and *in vivo* models

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Purpose/Objective: Inflammatory processes play a key role in various neurodegenerative diseases such as Multiple Sclerosis (MS), Alzheimer's Disease and Parkinson's Disease. Increasing evidence suggests that iron accumulation in the brain might contribute to neurodegeneration. Iron is a potential source of free radicals as it can catalyze the production of hydroxyl radicals under oxidative conditions. This can lead to amplification of tissue injury caused by oxidative damage. In preliminary experiments we found evidence that the presence of iron exacerbates H2O2 induced cell death in glial cells *in vitro*.

Materials and methods: We thus characterized iron storage within the central nervous system (CNS) of animal models of different neurodegenerative diseases. We examined animals with acute inflammation mediated by CD8 or CD4 positive T cells and animals suffering from T cell and antibody mediated chronic inflammation due to active immunization. Further, we studied LPS induced lesions which represent CNS disease caused by the innate immune system. Similarly, we characterized oxidative damage in the different models for inflammation.

Results: We did not find evidence for the presence of oxidized phospholipids or oxidized DNA in the experimental lesions, which is in contrast to our results of MS lesions. None of these models showed iron accumulation in glial cells comparable to what is seen in MS tissue. However, some iron positive microglia and perivascular macrophages were observed in acute and chronic models. As iron accumulates progressively with aging, we analyzed iron storage in aged rats. We observed that iron is stored in oligodendrocytes and myelin in certain areas of the brain such as the deep cerebellar nuclei and the basal ganglia. To address the question, if iron could influence CNS inflammation, we compared acute young and old animals. We found a similar disease course in both groups whereas in the pathological analysis we detected higher levels of inflammatory infiltrates in young animals. In contrast, we found a higher degree of neurodegeneration in the spinal cords of old animals. Nevertheless, we did not find any evidence for iron deposition in glial cells.

Conclusions: These observations necessitate the search for additional animal models mimicking the human situation more closely with respect to age dependent iron accumulation and neurodegeneration.

P1158

The Vagus nerve, importance in immunodepression following stroke

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Purpose/Objective: Impaired immune function due to overactivation of neuro-humoral stress pathways, in particular of the sympathetic nervous system, has been proposed as risk factor for the high incidence of pneumonia after stroke. However, changes in pulmonary immunity and the role of the parasympathetic vagus nerve (VN) in stroke-induced suppression of immune responses are poorly understood so far. We hypothesize that after stroke pulmonary macrophage function is impaired due to release of acetylcholine by the VN resulting in reduced lung anti-bacterial responses. This anti-inflammatory effect mediated by the VN is dependent on alpha7 nicotinic acetylcholine receptor (α7nAChR).

Materials and methods: To investigate the role of the VA in stroke-associated pneumonia we analyzed effects of vagotomy (Vtx) on cellular immune responses to bacterial pneumonia in an experimental model of cerebral ischemia (MCAo) in WT C57/BL6 mice. In addition, α7nAChR$^{-/-}$ mice and littermate controls were used to address the role of the α7nAChR in stroke-induced immunosuppression. To determine the role of the α7nAChR expression on pulmonary immune versus parenchymal lung cells we used bone marrow chimeras reconstituted with α7nAChR$^{-/-}$ and WT cells. The immune responses in lung and spleen were analyzed by flow cytometry, by multiplex cytokine analysis of BAL fluid and *ex vivo* TLR-ligand induced cytokine secretion, and assessment of macrophage phagocytic activity.

Results: Inhibition of VN activity by Vtx significantly ameliorated spontaneous bacterial infections after stroke in wt mice. Release of pro-inflammatory cytokines such as TNF-a by alveolar macrophages upon *ex vivo* stimulation with TLR-ligands was significantly increased in Vtx mice. Interestingly, our mixed bone marrow chimera experiments indicate that α7nAChR expression on lung parenchymal cells is also important for impaired pulmonary anti-bacterial responses mediated by increased VN activity after stroke.

Conclusions: We could show that the VN and the a7AchR play a crucial role in suppression of lung immunity after stroke. Inhibition of parasympathetic activity by Vtx or a7AchR deficiency reduces susceptibility to bacterial lung infection after acute CNS damage. Expression of a7AchR on both lung macrophages and parenchymal cells seem to be important to the immunosuppressive effect of increased VN activity after stroke.

Poster session: Parasitic Diseases

P1160

A role for plasmacytoid dendritic cells in promoting Th2 immunity against helminths

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Purpose/Objective: Plasmacytoid dendritic cells (pDC) are a distinct subset of dendritic cells that are found in the blood as well as in peripheral lymphoid tissues. While pDC are best known for their role in antiviral immunity (and their potent ability to produce type I interferons), they also appear to be involved in regulation of Th2 responses in allergy. However, it is not known how pDC respond to Th2-associated pathogens such as the parasitic helminth *Schistosoma mansoni*, and whether they play an immunogenic or tolerogenic role in Th2 infection settings.
Materials and methods: In this study, we have characterized the response of pDC in the liver effector site during *S. mansoni* infection, both in terms of their phenotypic activation and their ability to present antigen to naïve and effector/memory $CD4^+$ T cells. The mAb 120G8 was also used to determine the impact of pDC depletion on the development of Th2 immunity *in vivo*.
Results: Our results demonstrate that liver pDC recognise and respond to *S. mansoni* antigens by up-regulating surface expression of MHC class II and co-stimulatory molecules. Liver pDC also acquire the capacity to support the proliferation of antigen-experienced IL-4/IL-13 producing $CD4^+$ T cells during infection. Furthermore, in the absence of pDC, *S. mansoni*-specific $CD4^+$ T cell production of IL-4 and IL-13 was impaired in the liver.
Conclusions: Together our data provide strong evidence that liver pDC promote Th2 immunity against *S. mansoni* parasites.

P1161

A SAG2A protein-based algorithm to analyze the production of IgG1 and IgG3 subclasses in sequential serum samples from patients with acute toxoplasmosis

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Purpose/Objective: The major aim of the present study was to evaluate the kinetics of the humoral immune response to the recombinant SAG2A antigen in comparison with *T. gondii* soluble antigen (STAg) through the detection of specific IgG, IgG1 and IgG3 antibodies in serum samples of patients with toxoplasmosis in different time points of infection. Also, we investigated the association of these serological markers based on SAG2A reactivity as potential tools to distinguish early acute from convalescent phase of toxoplasmosis.
Materials and methods: It was carried out a prospective longitudinal study that evaluated a total of 130 serum samples obtained from 19 patients with acute toxoplasmosis after different time points of illness onset. The patients enrolled in the study were attended at the Infectious Disease Center of the Federal University of Espirito Santo, Vitoria, ES, Brazil, after an initial screening by physicians of the region. Serological evidence of recent infection was characterized by the presence of IgM and/or IgG antibodies to *T. gondii* in conventional serological assays using a commercial kit (ELFA, VIDAS® Toxo IgM and IgG II, Biom−rieux SA, Lyon, France). Exclusion criteria were pregnancy and/or human immunodeficiency virus (HIV)-positive patients. Indirect ELISAs for the detection of IgG antibodies and their subclasses (IgG1, IgG2, IgG3, and IgG4) to SAG2A and STAg were also performed.
Results: The follow up of IgM and IgA levels to STAg showed a gradual decrease, with the majority of patients (88%) seropositive for IgM up to 12 months of infection, whereas IgA seropositivity was relatively low (78%) compared to IgM (100%) in the first 3 months of infection. The follow up of IgG and IgG1 antibodies showed a similar increasing profile for both SAG2A and STAg, with slightly higher seropositivity for STAg. The kinetics of IgG3 to STAg was similar to that of IgG1, contrasting with the kinetics of IgG3 to SAG2A that showed high levels up to 6 months of infection, with continuous decreasing over the time. Higher IgG3 seropositivity to SAG2A than STAg was also observed in the initial phases of infection. A higher IgG3/IgG1 ratio for SAG2A than STAg was detected in the first 3 months of infection, with decreasing profile over the time.
Conclusions: Altogether, our results demonstrate a differential kinetics of IgG3 antibodies to SAG2A and STAg in patients with toxoplasmosis up to 12 months of infection. Also, the IgG3/IgG1 ratio to SAG2A in association with classical serological markers of acute phase could constitute valuable tools to distinguish early acute from convalescent phases of Toxoplasma gondii infection.

P1162

Activation of PPAR-alpha decreases *in vitro* nitric oxide production and increases mice susceptibility to *Toxoplasma gondii* infection

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Purpose/Objective: Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors, consisting of three known isoforms (α, β and γ), of which PPARα is the most studied. PPARs function as lipid sensors, regulating metabolism and immune response by acting in the inhibition of pro-inflammatory cytokines. *Toxoplasma gondii* infection induces a robust Th1 inflammatory response with excessive nitric oxide (NO) production. The role of PPARs in the gut inflammation caused by *T. gondii* in different susceptible mice lineages is still unclear and the aim of this work was to evaluate the expression and activation of PPAR-α and its role in the susceptibility to *T. gondii* infection.
Materials and methods: C57BL/6 mice were orally infected with cysts of *T. gondii*, ME-49 strain. The transcripts of PPAR-α were evaluated at 0, 4, 6 and 8 days post-infection (p.i.) by real time PCR (qPCR) of ileum and liver on mice inoculated with 40 cysts. One group of animals infected with five cysts was treated with PPAR-α agonist (Gemfibrozil) for mortality evaluation. NO production was assessed in the supernatant of splenocytes cultured for 48 and 72 h after *T. gondii* infection and agonist treatment *in vitro*.
Results: PPAR-α expression was downregulated in ileum and liver at day 8 p.i. when compared to the previous periods evaluated. Interestingly, when mice were treated with PPARα agonist at 10 mg/

kg/day during 7 days p.i. there was an increase on mortality. *In vitro* infection and agonist treatment showed that PPAR-α activation reduced NO production of C57BL/6 mice infected spleen cells.
Conclusions: These data indicate that PPAR-α is modulated by *T. gondii* infection with equals patterns of expression in ileum and liver in susceptible mice. Moreover, activation of PPAR-α induces an increase of mortality and susceptibility to infection besides a reduction in NO production caused by *T. gondii*. These results lead to a better understanding of the susceptibility to this infection and provide basis for future approaches aimed at controlling exacerbated gut inflammation.

P1163
Apoptosis as mechanism of supression of regulatory T cells in Chagas disease

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Purpose/Objective: The Chagas disease is the fourth most important tropical disease, which affects approximately 15 million people in Latin America. The mechanisms involved in the development of the severe forms of this illness are not well known yet. Recently, it has been observed an important protective role of regulatory Tcells in this disease, although its mechanism remains unclear. Thus, the aims of this study is to evaluate apoptosis as possible mechanism used by regulatory Tcells ($CD4^+$ $CD25^+Foxp3^+$) and characterize the profile cytokines in Chagas disease.
Materials and methods: To this end, the peripheral blood of 11 patients were collected and the analyzed by flow cytometry. The patients were grouped in the indeterminate (IND) and cardiac (CARD) clinical forms of Chagas disease. To evaluate the apoptotic profile, it was used monoclonal antibodies reactive to PD1, PD1L, CD39, CD95, CD95L, CTLA-4, GITR, CD107 and granzyme B conjugated with PERCP, APC, PE and FITC fluorochromes. The profile of cytokines was also evaluated through the staining of IL-1β, IL-17, TGF-β and IL-10 conjugated with PE fluorochrome.
Results: The results show no significative difference in the expression of CD95, CD95L, CTLA-4, GITR and granzyme B after comparison between patients of the different groups in the presence or absence of stimulus. However, it may be observed a higher expression of PD1 after stimulus in the IND group ($P = 1.000$) and CARD group ($P = 1.000$). In addition to a reduction in the expression of CD39 after stimulus in the IND group ($P = 0.312$) and CARD group ($P = 0.437$). CARD patients present higher expression of PD-1L ($P = 0.0173$) than IND patients in the absence of stimulus. Moreover, the regulatory Tcells of CARD patients present higher expression of CD107 after comparison with and without stimulus ($P = 0.0313$). No significative difference was observed in the expression of IL-1β and IL-10. However, it may be observed a reduction in the expression of IL-17 after stimulus in both groups IND ($P = 0.2087$) and CARD ($P = 0.3125$). Furthermore, IND patients present higher production of TGF-β by regulatory Tcells, If compared to CARD patients ($P = 0.1576$) in the absence of stimulus.
Conclusions: In conclusion, many mechanisms are involved in the apoptosis caused during the infection by *T. cruzi* and the presence of severe forms may be related to the fratricide of regulatory Tcells or the apoptosis of effector cells.

P1164
ArtinM, a D-mannose-binding lectin extracted from *Artocarpus integrifolia*, plays a potent adjuvant role in immunization process against *Neospora caninum*

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Purpose/Objective: *Neospora caninum* is a coccidian parasite, closely related to *Toxoplasma gondii*, with wide host range and worldwide distribution, causing neuromuscular disease in dogs and abortion or reproductive disorders in cattle. Neosporosis seriously impacts the dairy and beef industries leading to substantial economic losses. Cattle acquire the infection by horizontal transmission, through ingestion of food and drinking water contaminated with oocysts eliminated in faeces from canine definitive hosts; or more often, by transplacental vertical transmission, endogenous or exogenous, from an infected dam to her offspring during pregnancy. Vaccination is an important approach to control neosporosis, however, so far there is no effective vaccine to control this parasitic disease. ArtinM and Jacalin (JAC) are lectins from the jackfruit (*Artocarpus integrifolia*) that have important role in modulation of immune responses to pathogens.

The major aim of the presente study was to evaluate the adjuvant effect of ArtinM and JAC in immunization of mice against neosporosis.
Materials and methods: Six C57BL/6 mouse groups were subcutaneously immunized three times at 2-week intervals with Neospora lysate antigen (NLA) associated with lectins (NLA+ArtinM and NLA+JAC), NLA, ArtinM and JAC alone, and PBS (infection control). Animals were challenged with lethal dose of Nc-1 isolate and evaluated for morbidity, mortality, specific antibody response, cytokine production by spleen cells, brain parasite burden and inflammation.
Results: Our results demonstrated that ArtinM was able to increase NLA immunogenicity, inducing the highest levels of specific total IgG and IgG2a/IgG1 ratio, ex vivo Th1 cytokine production, increased survival, the lowest brain parasite burden, along with the highest inflammation scores. In contrast, NLA+JAC immunized group showed intermediate survival, the highest brain parasite burden and the lowest inflammation scores.
Conclusions: In conclusion, ArtinM presents stronger immunostimulatory and adjuvant effect than Jacalin in immunization of mice against neosporosis, by inducing a protective Th1-biased pro-inflammatory immune response and higher protection after parasite challenge.

P1165
B cell epitope screening on *Clonorchis sinensis* through in silco-prediction and peptide microarray analysis

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Purpose/Objective: Serological diagnosis of clonorchiasis with crude antigens of *C. sinensis* is hampered by the cross reactivity with other trematode. Therefore identification and the use of highly sensitive and specific antigens are essential for the improvement and standardization of the serological diagnosis of clonorchiasis. In this study, we have studied to find strong antigen of *C. sinensis* via B cell epitope screening.

Materials and methods: We selected 22 peptides on 52 antigenic proteins which were reported and identification in *C. sinensis* utilizing five epitope prediction tools (BepiPred, BCPred, FBCPred, AAPPred and Antigenic). These peptides ranged between 13 and 29 amino acids were chemically synthesized and conjugated with biotin respectively. The peptides were spotted onto microarrays and screening the reactivity against sera from clonorchiasis patients. Seropositive human serum samples ($n = 50$) were collected from egg positive cases and seronegative human serum samples ($n = 20$) were volunteer healthy human.

Results: From a total of 22 peptides contained in the microarray, nine peptides showed the antigenic reactivity against pooled sera from clonorchiasis patients. The positive peptides that reacted above the cut-off determined from volunteer healthy human. The highest of antigenic peptides were ProR1 and Cys7 derived from Proline rich protein and Cysteine proteinase, respectively. When the antigenicity of both peptides was analyzed with individual serum of clonorchiasis patients, the sensitivity showed different pattern.

Conclusions: In the current study, we have identified two antigenic peptides for effective serological diagnosis could useful for diagnostic development. Our study provides the proof that the bioinformatic prediction and peptide microarray analysis are valuable tools for the discovery of *C. sinensis*-derived epitopes as serodiagnostic antigens with sufficient sensitivity and specificity.

P1166
B-1 cells increase susceptibility to murine visceral leishmaniasis

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Purpose/Objective: Leishmaniasis belongs to a group of diseases caused by protozoan parasites from *Leishmania* genus. The immune response to leishmaniasis is complex and the result of infection depends both on the genetic composition of different species of *Leishmania* and the host immunity. Clinical and experimental evidences suggest that activation of B cells leads to exacerbation of visceral leishmaniasis and contributes to susceptibility of cutaneous leishmaniasis. B-1 cells are a subtype of B lymphocytes whose role in the physiology of the immune system as well as in the pathogenesis of various diseases is still poorly understood. These cells produce large amounts of IL-10 cytokine which plays a key role in immunosuppression in several diseases such as leishmaniasis. In this study we investigated the role of B-1 cells in the pathogenesis of visceral leishmaniasis.

Materials and methods: Experiments were performed with BALB/c, BALB/*Xid* or BALB/*Xid* mice that receive adoptively peritoneal B-1 lymphocytes. These groups were infected or uninfected with *L. (L.) chagasi*.

Results: Our results showed that BALB/*Xid* mice, a mouse strain deprived of B-1 cells, infected for 45 days with *L. (L.) chagasi* had a significant reduction in parasite load in the spleen ($0.63 \times 10^7 \pm 0.4$ parasites/mg tissue) when compared to control animals BALB/c ($1.87 \times 10^7 \pm 0.6$ parasites/mg of tissue) or BALB/*Xid* that received adoptive transfer of B-1 cells ($1.44 \times 10^7 \pm 0.5$ parasites/mg tissue). These data were also confirmed by qPCR. Flow cytometry analysis demonstrated changes in peritoneal B-1 cell population. Infected BALB/c mice showed a significant increase in the percentage of peritoneal B-1 cells (70.0% ± 1.50) compared to control (63.3% ± 1.88). On the other hand, BALB/*Xid* that received adoptive transfer of B-1 cells and were infected with *L. (L.) chagasi* showed a significant decrease in the percentage of peritoneal B-1 cells (6.14 ± 3.01%) when compared to uninfected group (25.48 ± 7.60%). The IL-10 production was evaluated in supernatants from spleen homogenates of uninfected and infected mice. No differences were detected between BALB/c uninfected and infected group. However, BALB/*Xid* that received adoptive transfer of B-1 cells and were infected with parasite had higher levels of IL-10 when compared with unifected mice.

Conclusions: Our results suggest a possible role of B-1 cells in susceptibility to *L. (L.) chagasi*.

P1167
CD8+ T cell epitopes: a new source for vaccination against cutaneous *Leishmania major* infections

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Purpose/Objective: Infections with the parasite *Leishmania* (L.) *major* are caused by the bite of a sandfly. Healing is eventually based on IFNg secretion by $CD8^+$ and $CD4^+$ T cells. However, it is still unclear, which peptides finally promote protection and long-lasting immunity against infections with *L. major*. Up to date, no vaccine against this human pathogen exists. Thus, we are highly interested in identifying peptides that mediate healing of *L. major* infections.

Materials and methods: The most abundant proteins of both life-forms were identified by mass spectrometry. Subsequently, from these proteins, epitopes were predicted using the computer-based algorithm SYFPEITHI. 300 K^b/D^b peptides were chosen based on their predicted immunoreactivity. Additionally, all peptides were analysed for their binding-affinity towards MHC class-I molecules. A correlation between the predicted and meassured binding affinity of the peptides towards MHC class-I molecules was observed. Further, all 300 peptides were tested in *in vitro* assays. To this aim, peptide-loaded dendritic cells of C57BL/6 mice were cocultured with primed $CD8^+$ T cells for 48 h.

Results: Supernatants were analysed for their IFN-g, IL-4 and IL-10 content. Interestingly, we identified ~20 peptides with induction of high amounts of IFN-g and low levels of IL-4 and IL-10. Based on their cytokine profile, we randomly chose 16 peptides for further *in vivo* experiments. Here, C57BL/6 mice were immunized either in a prime/boost (P/B) (10 μg peptide twice) or a prime/boost/boost (P/B/B) (20 μg/2 × 10 μg peptide) approach in one ear. Three weeks later, mice were infected with 1×10^3 metacyclic promastigotes in the alternate ear. Lesions volumes were assessed weekly. Surprisingly, five peptides partially protected mice against challenge using the P/B approach compared to PBS-control mice. This effect even increased, when mice were immunized in the P/B/B approach.

Conclusions: In summary, identifying peptides which protect mice against challenge with *L. major* would finally lead to a long desired vaccine against this human pathogen. Further, tetramer development with *L. major* specific peptides would aid the understanding of T cell priming during parasitic infection.

P1168

Characterization of the virulence of *Leishmania infantum* isolates from human patients

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Purpose/Objective: Leishmaniasis is a group of diseases with a variety of clinical manifestations. The severity of the disease is highly dependent on the immunological status and genetics of the host. The virulence of the species/strain also plays an important role in the progression of the infection. In this study we have characterized some strains of *L. infantum* in terms of *in vitro* cultivation and differentiation through the life cycle of promastigotes and compared their virulence and infectivity *in vivo*.

Materials and methods: *L. infantum* strains used in this study were isolated from 2 HIV/*Leishmania*-coinfected patients and 1 immunocompetent patient with cutaneous disease. We defined the best culture conditions to grow the parasites, having tested five well-established culture media that differ in their complexity and nutrients availability. Growth curves, viability analysis, cell cycle progression and metacyclogenesis-specific Meta-1, SHERP and Histone H4 relative expression were evaluated. In adition, *Leishmania* infections in bone marrow-derived macrophages were executed and final assays of virulence were performed in Balb/c mice.

Results: From the culture media tested, NNN medium demonstrated to maintain viability and to generate stationary promastigotes, allowing the differentiation into metacyclic forms for all strains. *In vitro* infections showed one of the tested strains to be more infective and resistant to the hostile intracellular environment of the macrophages. We have confirmed this result with Balb/c mice infections, where this differential virulence was maintained in acute and chronic infections. In addition, other assays were carried out using mice previously infected with each strain and challenged with the most virulent strain. Mice infected with least virulent strains showed high parasite loads in spleen, liver and bone marrow; however animals inoculated and reinfected with the most virulent strain induced lower parasite loads and a protective response.

Conclusions: In conclusion, this work has shown an efficient approach to characterize the virulence of *Leishmania infantum* strains and to study some of the underlying mechanisms that makes a host to be capable of control *Leishmania* infection.

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P1169

Chronic infection drives co-expression of the inhibitory receptor CD200R, and its ligand CD200, in mouse and human T cells

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Purpose/Objective: Certain parasites evade the immune response and establish chronic infections that may persist for years. Under these conditions, upregulation of surface receptors with inhibitory properties regulate immune cellular activation and associated pathology. The negative regulator, CD200 receptor, and its ligand, CD200, regulate macrophage activation and reduce pathology in viral infections. Little is known about the modulation of CD200:CD200R in T cells and during chronic helminth infection.

Materials and methods: T cells were either transiently or chronically stimulated with anti-CD3/CD28 and peptide, *in vitro*, or recovered from different organs (liver, spleen and mesenteric lymph nodes) of chronically infected mice with the trematode, *Schistosoma mansoni* (80 Cercariae, 8 weeks post-infection). T cell expression of CD200: CD200R was investigated alongside expression of activation markers (CD44, CD25 and CD69) and secretion of cytokines (IL-2, TNFa, IFNg and IL-4) following 5 h-re-stimulation with PdbU and Ionomycin by flow cytometry. Additionally, expression of CD200R in T cells in PBMC from individuals endemically exposed to Schistosoma heamatobium was correlated to cytokine secretion and parasite egg counts in the urine.

Results: T cells co-express CD200 and CD200R after TCR stimulation. Sustained expression of CD200R required chronic rather than transient TCR stimulation and coincided with loss of multifunctional potential (IL-2 and TNFa) and terminal effector differentiation in T cells. Terminally differentiated CD4 T cells increased expression of CD200R in response to chronic helminth infection, with *Schistosoma mansoni,* particularly in organs of pathogen persistence, where Th-2 effectors accumulate in infection. Similarly, in humans infected with *Schistosoma haematobium*, we detected an association between IL-4 production and CD200R expression on T effector cells that correlated effectively with egg burden and, thus infection intensity.

Conclusions: CD200:CD200R in T cell responses to helminths has diagnostic and prognostic relevance as a marker of infection for chronic schistosomiasis in mouse and man. CD200R efficiently marks terminally differentiated effectors. We suggest that interfering with CD200:CD200R may recover functional properties of Th2 effectors.

P1170

Evaluation of IL-17 expression in human Chagas disease

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Purpose/Objective: This study was designed to investigate whether the expression of IL-17 is associated with the no apparent heart disease or cardiomyopathy clinical forms of Chagas disease.

Materials and methods: It was employedacross-sectionaldesigninvolvingpatientsfromendemicareaswithin MinasGerais, Brazil, underthe medicalcareofone of us (MOCR). Positive serology for Chagas disease was determined by two or more different tests. The patients infected with *T. cruzi* were grouped as no apparent heart disease (NAHD) and cardiomyopathy (CARD) patients ranging in age from 18 to 65 years old. The NAHD group included 82 individuals, with no significant alterations in electrocardiography, chest X-ray and echocardiogram. All 94 CARD patients presented with dilated cardiomyopathy, characterized by the echocardiographic finding of a dilated left ventricle with impaired ventricular systolic function. A total of 24 Normal healthy individuals, ranging in age from 29 to 55 years old, from a non-endemic area for Chagas disease and showing negative serological tests for the infection were included as a control group (NI). A cytometric bead array (CBA) immunoassay kit (BD Biosciences, USA) was used for the analysis of plasmatic cytokines, including IL-17. A second fluorescently labeled PE-anti-cytokine antibody was added and the concentration of the individual cytokines was indicated by their Mean Fluorescence Intensity (MFI). Data were acquired in a FACScalibur flow cytometer (Becton Dickinson-BD) and the analyses were performed using BD CBA software.

Results: It was compared the three groups with relation to the expression of IL-17. The Kruskal-Wallis test was used with the significance of 5%. The hypothesis of equality between the groups was rejected. The comparison was performed between groups of two. It was used the Wilcoxon test with Bonferroni correction (significance level = 0.05/3 = 0.0167). It was observed difference between the groups CARD and NAHD (P-value < 0.001) and between NI and NAHD (P-value = 0.008). There was not significative difference between the groups CARD and NI (P-value = 0.376). The expression of IL-17 was higher in patients of the NAHD group, If compared with patients in the other groups, median of 11.81 (5.79−22.86) versus 5.38 (3.87−10.55) of CARD group and 5.715 (4.25−11.31) of the NI group.
Conclusions: Therefore, IL-17 expression seems to be associated with a protective cardiac function in human Chagas disease.

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Evaluation of serum cytokine concentrations of patients in different clinical stages of Chagas' disease

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Purpose/Objective: The aim of this study was to evaluate serum concentrations of cytokines IL-4, IL-10, IL-12, TNF-α and IFN-γ in patients in different clinical forms of Chagas' disease.
Materials and methods: We conducted a case-control study of 115 individuals. Infected individuals were divided into four stages as the Brazilian consensus of Chagas' disease: indeterminate patients or without apparent heart disease (normal electrocardiogram and echocardiogram) cardiac patients on the stage A (electrocardiogram changes and normal echocardiogram), cardiac patients on the stage C [electrocardiogram and echocardiogram altered with compensable congestive heart failure (CHF)] and cardiac patients on the stage D (electrocardiogram and echocardiogram changed with refractory CHF). Also were included uninfected individuals with *T. cruzi*. We included 30 indeterminate patients, 31 cardiac patients on the stage A, 14 cardiac patients on the stage C, 11 cardiac patients on the stage D and 29 uninfected individuals.
Results: Among the pro-inflammatory cytokines, IFN-γ showed higher serum levels in relation to IL-12 and TNF-α. The cardiac patients on the stage A had higher concentrations of TNF-α, however, there was a significant decrease in the concentrations of this cytokine in the same time that we observed the later stages of the chronic Chagas cardiomyopathy (CCC). Both indeterminate and cardiac patients showed high levels of TFN-α and IFN-γ and low levels of IL-4 and IL-10, demonstrating a dominant Th1 profile with an imbalanced immune response.
Conclusions: This study demonstrated a direct proportionality in concentrations of pro-inflammatory and anti-inflammatory cytokines with respect to the left ventricular ejection fraction in all groups of patients, suggesting that this correlation could be used as a marker of progression to CCC.

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IgG and IgM anti-toxoplasma gondii antibodies class in high risk pregnant women in Brazil

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Purpose/Objective: Toxoplasmosis is a zoonosis caused by *Toxoplasma gondii*, an apicomplexa obligate intracellular protozoan parasite, which infects different species including humans. Fetuses can be infected by transplacental transmission, a condition that may cause significant sequelae in babies. The knowledge of the antigenic profile of IgM and IgG anti-*T. gondii* antibodies during the gestational period gives clinical support towards preventing the fetal infection by this parasite. This study aimed to verify the serological profile of the pregnant women from S'o Jos* do Rio Preto, S'o Paulo State, Brazil, which received medical attention in a public health service from a tertiary school hospital.
Materials and methods: The medical records of 793 pregnant women, attended in the High Risk Antenatal Care and Fetal Medicine, Gynecology and Obstetrics Outpatient Clinic of the Hospital de Base in S'o Jos* do Rio Preto, S'o Paulo State, Brazil, between 2001 and 2004, were analyzed. The serology tests performed to determine specific IgG and IgM anti-*T. gondii* antibodies were based in a commercial immunoenzymatic assay kit (ETI − TOXOK * IgG, ETI − TOXOK * IgM DiaSorin, Italy).
Results: From the overall data, 503 (63.4%) were reagent and 290 (26.6%), non reagent. Among the reagent pregnant women, 32 (4.0%) presented a serology profile with both anti-*T. gondii* antibodies IgG and IgM positive and 471 (59.4%), with anti-*T. gondii* antibodiesIgG positive and IgM negative. The profile of anti-*T. gondii* antibodies IgG negative and IgM positive was not found.
Conclusions: The prevalence of *T. gondii* infection is high in the region where this study was carried out and the majority of the pregnant women with positive serology tests are not under risk of gestational transmission of this parasite. However, considering that 4.0% of the pregnant women are under risk of gestational transmission, these results attract the attention for the necessity to implement a protocol for serological and molecular screening of mother and newborn babies, to contribute with the clinical guidance and to prevent babies sequelae.

P1173

IL-10 secreting, type 1 regulatory T cells and naturally occurring regulatory T cells differently modulate IgG secretion by B cells in human hypo-responsive onchocerciasis

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Purpose/Objective: Onchocerciasis or river blindness is the second leading infectious cause of blindness after trachoma. The disease is caused by infections with the filarial nematode *Onchocerca volvulus* and usually present two distinct pathological forms: the hyper-reactive or Sowda form and the hypo-reactive or generalized form. The hypo-reactive form is characterized by anergy and tolerance to the parasite,

whereas the Sowda form is associated with strong local immune reactions leading to immunopathology. The cellular and molecular patterns associated with this polarization of the disease are still not fully clarified. The present study has focused on the capacity of Tr1 and Foxp3 expressing regulatory T cells Tregs isolated according to their expression of CD25 alone, CD25 and CD127, and CD25 and CD49d, to modulate B cells antibody secretion in the presence or absence of additional B cell activating signals.

Materials and methods: The different cell subsets were isolated from filarial-infected individuals or healthy blood spenders, using magnetic cell isolation. Purity and cell phenotype were assessed by FACS analysis. Cytokines and antibody secretion were analyzed by ELISA and cytometric bead array.

Results: We could show that, the secretion of the non-cytolytic immunoglobulin IgG4 constitutes a humoral signature, associated with hyporesponsiveness and tolerance in human onchocerciasis. Using a sequential depletion-reconstitution strategy on peripheral blood mononuclear cells from patients with the generalized form of onchocerciasis, we could also demonstrate that, both Tr1 and Treg cells are involved in this IgG4 secretion. However, direct co-cultures of Tr1 and Tregs with autologous B cells reveal significant differences in the modulation of B cells antibody secretion by the two regulatory T cell types. While Tr1 are capable of activating B cells and preferentially induce IgG4 secretion, purified Foxp3 expressing $CD4^+$ $CD25^+$ regulatory T cells only weakly induced IgG4 secretion. Remarkable differences were also observable depending on the methods used for Treg purification. While $CD4^+$ $CD25^+$ Tregs slightly induced IgG4 secretion, $CD4^+CD25^+CD127$dim and $CD4^+CD25^+CD49d^-$ Tregs inhibited B cells activation and antibody secretion by reducing B cell proliferation, survival and maturation into plasma cells.

Conclusions: These findings confirm the implication of both IL-10 secreting regulatory T cells and $CD25^+$ Tregs in IgG4 induction during generalized onchocerciasis. Our results also suggest a direct role for Tr1 cells in IgG4 secretion by B cells, whereas $Foxp3^+$ Tregs inhibit IgG secretion and only indirectly promote IgG4 secretion.

P1174

IL-7 reverses T cell hypo-responsiveness in chronic schistosomiasis

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Purpose/Objective: Schistosomiasis is a major cause of morbidity worldwide. Infected individuals are susceptible to re-infection and lack effective T cell memory. The T cell response against schistosomes is a combination of activation, hypo-responsiveness/anergy and strong immunoregulation. IL-7 plays non-redundant roles in both B and T cell biology, crucially regulating thymopoiesis, naïve T cell survival/homeostasis, generation/maintenance of memory T cells, transition from effector to memory, and tumor-associated T cell exhaustion. We thus investigated whether IL-7 reverses T cell hypo-responsiveness in chronic schistosomiasis.

Materials and methods: T cells were recovered from different organs (liver, spleen, mesenteric and subcutaneous lymph nodes) of naïve and chronically infected mice with increasing dose of *Schistosoma mansoni* cercariae (8 weeks post-infection) and cultured with IL-7. Applications of IL-7 were tested during schistosomiais (7–8 weeks). T cell activation, polarization, exhaustion and hypo-responsiveness were evaluated by surface marker expression and secretion of cytokines (IL-2, TNFα, IFNγ, IL-5 and IL-4) following 5 h-re-stimulation with PdbU and Ionomycin by flow cytometry or in ELISA assays, upon SEA and anti-CD3-restimulation.

Results: In chronic schistosomiasis, depending on pathogen burden, T cells are widely activated leading to thymic atrophy accompanied by severe reduction in T cell numbers in lymphoid organs distal from the site of persistent infection. Activated cells localizing in the site of antigen persistence differentiate into effector/effector memory cells, with a reduction in less differentiated central memory CD4 T cells. These effector T cells show typical aspects of exhaustion (PD-1, CD200R), apoptosis (low Bcl-2) and hypo-responsiveness, while maintaining IL-7Rα expression. Application of IL-7 led to an increase in effector T cell cytokine responsiveness to schistosome antigens.

Conclusions: Chronic schistosomiasis leads to T cell polarization and exhaustion in the sites of parasite antigen persistence. We suggest that IL-7 may be useful to recover functional properties of Th2 effector cells and may be involved in regulating immunopathology.

P1175

Acute and chronic immune response in *Leishmania infantum*-infected Rhesus macaques. A novel model for visceral leishmaniasis

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Purpose/Objective: Visceral leishmaniasis (VL) is a chronic infectious disease caused by the protozoan *Leishmania infantum* or *L. donovani*. Rodent models have been widely used to dissect the immune response developed during VL. However, significant variations between these and human disease claim a shift towards new models capable of accurately mimicking human VL. We developed a non-human primate model for VL in Rhesus monkeys (RM) to dissect parasite dynamics and counteractive immune responses developed during acute and chronic infection.

Materials and methods: RMs of Chinese origin were intravenously inoculated with *L. infantum* (2×107 parasites/kg of body weight). Infected animals were subdivided in three groups that were sacrificed at day 11 post-infection (p.i.) (group 1), day 28 p.i. (group 2) and day 250 p.i. (group 3) for internal organ removal and analysis. Additionally, blood was sampled from remaining alive animals at days 7, 14, 21, 60 and 180 p.i.

Results: Quantification of parasite loads revealed the presence of parasites in the spleen, blood, liver and bone marrow early after infection. Parasite dissemination to lymph nodes (LN) was observed at the chronic phase concomitant with an increase in parasite loads in the blood and visceral organs. Early infection was characterized by splenic CD4 and CD8 T cell differentiation towards effector-memory phenotypes accompanied by increased sensibility to FasL-mediated cell death. Increased transcript levels of splenic IL-21 and IFN-γ were noticed at the acute phase. We demonstrated by intracellular cytokine staining the transient expansion of IL-21+ expressing CD4+ T cells associated with the production of specific IgM and IgG antibodies towards L infantum antigens. Consistent with the absence of parasite detection in peripheral LNs during acute phase, CD4 T cell differentiation to effector cells was modest without significant modifications on the cytokine microenvironment. In clear contrast, at the chronic phase, we observed decreased transcript levels of IFNγ and TNFα and increased IL-10 in both spleen and LNs.

Conclusions: Collectively, our findings point out the existence of a complex response to *L. infantum* infection in RMs with an early reaction of the host to infection, that includes expansion of IL21+ CD4 T cells, severely restricting parasite survival and an ability of the pathogen to colonize additional organs such as the LNs at chronic stages.

P1176

In *Leishmania major* infections the presence of leukocytes on C57BL/6 or BALB/c background is sufficient to induce a C57BL/6 or BALB/c phenotype, whereas stromal cells are not

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Purpose/Objective: Susceptibility to *Leishmania major* in BALB/c mice the result of predominant Th2 development, whereas C57BL/6 mice show a protective Th1 response. Previously, we reported that this is * in part − a result of differences in DC cytokine production, e.g. IL-1α/β and IL-12p80 homodimer. Others demonstrated the importance of cytokine production by stromal cells, e.g. keratinocytes. Applying a bone marrow chimera model we aimed to determine the key cell population(s) responsible for this strain-dependent dichotomy.
Materials and methods: Irradiated mice were injected with 5×10^6 bone marrow (BM) cells. Six to 10 weeks later, hematopoietic engraftment was analyzed by flow cytometry of blood samples. Reconstituted mice were infected with 2×10^5 *L. major* promastigotes intradermally into both ears and lesion development was measured weekly.
Results: To investigate this we used BALB/cxC57BL/6 F1 mice reconstituted with either BALB/c or C57BL/6 BM. Interestingly, the course of disease in infected mice reflected the origin of the BM: Mice reconstituted with BALB/c BM showed rapid disease progression, whereas mice reconstituted with C57BL/6 BM developed self-healing lesions. To further narrow down the key cell population(s) causing this effect, we applied a mixed bone marrow chimera model with mice differing only in T and B cells being either of BALB/c or C57BL/6 origin. Everything else, including the hematopoietic compartment, was uniformly BALB/cRag1$^{-/-}$ × C57BL/6Rag1$^{-/-}$ F1. These mice exhibit a disease phenotype according to the lymphocyte origin. Preliminary data shows that this holds also true in mice with C57BL/6 lymphocytes and the rest of the hematopoietic cells being of BALB/cRag1$^{-/-}$ origin (and *vice versa*).
Conclusions: These experiments showed clearly, that the presence of BALB/c-derived lymphocytes is not protective, while the presence of C57BL/6 lymphocytes is sufficient for protection. To further investigate the relevance of the genetic background of individual cell populations in this important disease, we have reconstituted mice leading to the concomitant presence of C57BL/6 T and BALB/c B cells and *vice versa*. Overall, the method of mixed bone marrow chimeras offers various opportunities to test the effects of individual cell populations in *L. major* infections as well as several other diseases.

P1178

Induction of IL-10-producing CD1dhighCD5$^+$ regulatory B cells following *Babesia* microti-infection

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Purpose/Objective: Understanding the induction of immune regulatory cells upon helminth infection is important for understanding the control of autoimmunity and allergic inflammation in helminth infection. *Babesia microti*, an intraerythrocytic protozoan of the genus *Babesia*, is a major cause of the emerging human disease babesiosis, an asymptomatic malaria-like disease. We examined the influence of acute infection by *B. microti* on the development of regulatory B cells together with regulatory T cells.
Materials and methods: To establish a well-defined animal model for *B. microti* infection, we monitored Babesiosis during the acute phase of infection. To confirm successful infection, spleens were weighed and parasitemia was calculated. Next, to determine whether *B. microti* infection selectively induces Bregs, we analyzed the expression of cell surface markers on various splenic B cell subpopulations in uninfected and infected mice. Flow cytometry was performed to examine the frequency of IL-10-secreting CD1d^{high}CD5$^+$ B cells and CD4$^+$ CD25$^+$ FoxP3$^+$ Tregs in the spleen of *B. microti*-infected mice.
Results: *B. microti* infection induced interleukin-10-producing CD1d^{high}CD5$^+$ regulatory B cells. Moreover, the CD4$^+$ CD25$^+$FoxP3$^+$ T cell frequency was increased significantly during the course of *B. microti* infection. After infection with *B. microti*, a high level of IL-10 was detected in the serum throughout the experiment.
Conclusions: This study is the first demonstration of the expansion of immune regulatory cells such as regulatory B cells following infection by an intraerythrocytic protozoan parasite. These data suggest that *B. microti* infection in mice provides an excellent model for studying Breg-mediated immune responses, as well as indirectly elucidating the mechanism by which helminth infection regulates autoimmunity and allergic inflammation.

P1179

Insight in the intestinal immune response during a Giardia muris infection in BALB/c mice

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Purpose/Objective: The protozoan parasite *Giardia duodenalis* is one of the most commonly found intestinal pathogens in humans and animals. Previous work on *Giardia* infected calves revealed an upregulated expression of the peroxisome proliferator-activated receptors (PPAR) in infected animals. The upregulation of these transcription factors and their anti-inflammatory capabilities could possibly explain the chronic nature of a *Giardia* infection. The aim of this study was to further unravel the role of these transcription factors in the host response using a *Giardia muris* − mice infection model.
Materials and methods: In a first trial, the intestinal immune response in BALB/c mice was investigated 1, 2 and 3 weeks after an infection with *G. muris*. Cyst secretion in the faeces was counted daily and intestinal gene expression levels for a panel of cytokines and PPARs were examined using qRT-PCR. In a second trial, the effect of a PPARα agonist (fenofibrate) on the intestinal immune response during a *Giardia* infection was analysed and compared to infected control mice and mice just receiving the agonist. Identical as in experiment 1, cyst secretion was monitored daily and changes in gene expression levels were measured by qRT-PCR.
Results: Analysis of the cytokine response in trial 1 showed a major upregulation of IL17A from 1 week p.i. onwards. The highest upregulation was observed at week 3, coinciding with a drop in cyst secretion. In contrast to the situation in calves, no transcriptional changes were observed for the PPARs. Identical as in trial 1, IL17 expression was also significantly upregulated in all the infected animals of trial 2, with the highest levels found in the infected animals treated with the fenofibrate. Analysis of the PPARs showed that, in contrast to trial 1, a modest upregulation of PPARα expression was observed in the *Giardia* infected control mice. Although this upregulation was not further induced by the fenofibrate treatment, animals receiving the fenofibrate shedded significantly less cysts compared to control animals during the first 3 weeks of an infection.
Conclusions: The outcome of this study suggests that IL17 plays an important role in the protective immune reponse against *G. muris* in mice. The role of PPARα in the immune response is still unclear and needs further research.

P1180

Investigation into the metabolic profile and transcriptional regulation of alternatively activated macrophages during helminth infection

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Purpose/Objective: Chronic inflammation is the hallmark of many diseases, in particular those associated with the metabolic syndrome. A growing body of evidence links alterations in cellular metabolism with inflammation, in particular classical macrophage activation with glycolysis. *In vitro* studies have defined a role for mitochondrial lipid metabolism, and the transcription factor PPARγ, in maintaining anti-inflammatory alternatively activated macrophages (AAMΦ). Using the helminth infection model *Brugia malayi*, a potent inducer of AAMΦ, we sought to investigate the role of lipid metabolism in AAMΦ *in vivo*.
Materials and methods: We obtained MΦ from wild type or IL4R$\alpha^{-/-}$ mice 21 days after *B. malayi* (NeMΦ) infection, or 3 days post thioglycollate injection (ThioMΦ). MΦ were FACS purified and RNA-Seq was performed. After gene expression quantification and differential expression analysis, coordinately regulated metabolic pathways were identified using GSEA. Transcription start sites were identified using a novel method, and over-represented transcription factor binding sites (TFBSs) were assessed using Clover. Mass spectroscopy was used to identify MΦ-derived eicosanoids elicited in the same manner as for the RNA-Seq experiment.
Results: NeMΦ up-regulated mitochondrial gene expression *in vivo*. Surprisingly NeMΦ expressed very low levels of PPARγ, but did express PPARδ. Analysis of *cis*-regulatory features in AAMΦ promoters revealed an over-represented PPAR response element. Finally, lipidomic profiling of macrophage-derived eicosanoids showed that AAMΦ abundantly produce the PPARδ ligand prostacyclin (PGI2).
Conclusions: We show that NeMΦ demonstrate enhanced mitochondrial metabolism, in particular the TCA cycle. Furthermore, the over-represented PPAR motif in AAMΦ promoters provides evidence for PPAR-dependent gene expression *in vivo*. Although PPARγ expression levels are low in NeMΦ, we show that NeMΦ abundantly produce the PPARδ ligand PGI2 providing a mechanism for PPAR-dependent transcriptional regulation in NeMΦ *in vivo*.

P1182

LL37 specific killing of *Leishmania* parasites inside human macrophages

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Purpose/Objective: Leishmaniasis is induced by promastigote parasites. Inside macrophages (MF) parasites change into multiplying amastigotes, propagating disease. There are different phenotypes of human MF, such as inflammatory type I (MF I) and anti-inflammatory type II (MF II) and it is not yet clear which phenotype of MF is involved in parasite propagation or killing. In a murine model for *Leishmania* infection it was demonstrated that parasites can be killed in an iNOS dependent manner.
Materials and methods: For human MF it is unclear which anti-parasitic mechanisms are used to control *Leishmania*. In this study we search for MF phenotype specific control mechanisms of both *Leishmania major* (*L. major*) promastigotes and amastigotes.
Results: We found that MF II are more susceptible to infection as compared to MF I. Characterizing both phenotypes in more detail revealed the antimicrobial peptide LL37 is up-regulated in MF I as compared to MF II. LL37 is shown to be involved in the innate host defence against various pathogens. Consistent with this we found that recombinant human LL37 can kill *L. major* parasites, extracellularly. To demonstrate its intracellular activity against *Leishmania*, we established a siRNA knockdown for LL37 in primary human MF. Subsequently, we found that a LL37 knockdown in MF I resulted in a significant higher promastigote survival, as compared to control MF I.
Conclusions: In conclusion, we demonstrate that LL37 is able to specifically kill *L. major* promastigotes, not amastigotes, in human MF I. Interestingly, the lack of anti-leishmania LL37 activity in MF II suggests them to be more suitable host cells.

P1183

Lymphoid organs behavior and dinamic of lymphocyte populations in *Trypanosoma cruzi* infected mice by oral or intraperitoneal route

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Purpose/Objective: To analyze subcutaneous lymph nodes, mesenteric lymph nodes, spleen and Peyer patches behavior, dynamic of lymphocyte populations and cytokine production in experimental Chagas' disease infected by oral/intragastric (IG) or intraperitoneal (IP) route.
Materials and methods: BALB/c mice were infected with 5×10^4 *Trypanosoma cruzi* trypomastigotes IP or IG and parasitemia was followed. After 9, 12, 16, 27 days, subcutaneous (SCLN) and mesenteric lymph nodes (MLN), spleen (Sp) and Peyer patches (PP) were harvested for cell counting and flow cytometry. Blood samples were collected for cytokine detection in 3, 9 and 12 days post-infection (dpi). Hearts were harvested at 15 dpi and histological analysis performed.
Results: IG infected mice presented lower parasitemia and mortality than IP infected mice. Parasites appeared within the blood after 3 days in IP mice and 9 dpi, in IG mice. In IP infection, 95% of mice died before 18 dpi, while in IG group, 90% were still alive 27 dpi.SCLN and Sp showed increased number of cells in both groups; however, hypertrophy of these tissues as well as T and $CD19^+$ cells expansion was less evident IG mice than IP. In mucosal-associated lymphoid tissues, such as PP, IP infection promoted atrophy after 12 dpi, due to decrease in $CD19^+$ and $CD4^+$ cells. In IG, PP cell number and T and $CD19^+$ cell depletion occurred in later stages of infection. MLN atrophy was only observed in IG group after 16 dpi, due to depletion of $CD19^+$ cells. Serum analysis of IP infected mice showed a Th1 bias, with increased production IFN-γ and TNF-α compared to IG and controls. Interestingly, IG group seems to be Th2 biased, with higher IL4 production than IP. Heart histological sections stained with Hematoxilin and Eosin showed alterations in 15 dpi (parasite nests, inflammatory infiltrate and tissue damage) were similar in both groups, even with the lower number of circulating parasites in IG mice.
Conclusions: Our results indicate that the route of *T. cruzi* infection is a key factor to stimulate the immune response against the parasite in the host.

P1184

Motor behavior dysfunction, neuronal death and glial activation in IL-12p40KO mice that were infected with *Trypanosoma cruzi*

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Purpose/Objective: Neurological disorders have been described in children and in immunosuppressed hosts with Chagas' disease, a Latin America protozoosis caused by *Trypanosoma cruzi*. Recently we showed that IL-12p40KO mice that were infected by *T. cruzi*, but not infected wild-type (WT) mice, develop a progressive paralysis that culminates in death.

Objective: To analyze the elements involved in the neurodegenerative process presented by *T. cruzi*-infected IL-12p40KO (KO) mice.

Materials and methods: KO and WT mice were infected by *T. cruzi* Sylvio X10/4 and submitted to behavioral analyses. Spinal cords (SCs) of both groups were analyzed every week by RT-PCR to estimate the kinetics of parasitism and immune transcripts, or analyzed by immunohistochemistry when KO mice presented complete paralysis to quantify neurons, astrocytes, macrophages/microglias, *T. cruzi* and other parameters.

Results: Eye-seen and computerized behavior evaluation revealed that KO mice developed a progressive paralysis, from the tail to the forelimbs (p *T. cruzi* amastigotes, most of them inside macrophages/microglias. No lesions were observed in WT SCs. Compared to WT, KO SCs showed increased immunoreactive area for nitrotyrosine (239%, p *T. cruzi* 18S rRNA transcripts revealed the parasite was controlled in WT SC but kept increasing in KO SC (p *T. cruzi* transcripts were significantly lower in WT SCs.

Conclusions: IL-12 and IL-23 efficiently act constraining *T. cruzi* parasitism in the CNS, occurring exacerbated inflammation and neurodegeneration in their absence.

P1185

Mucosal barrier attenuates exaggerated inflammatory responses to toxoplasma but does not restrict pathogen dissemination to the brain

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Purpose/Objective: Toxoplasma enters the host mostly through the oral route, overcomes local and systemic host resistance and passes mucosal and blood-brain barriers in order to reside in brain. As the mechanisms restricting pathogen entrance to the brain are not clearly understood the aim of this study was to compare the dissemination of toxoplasma in intraperitoneally (ip) and orally infected mice and local immune responses that accompany systemic and mucosal route of infection.

Materials and methods: A/Snell mice were infected with 10^5 tachyzoites of *T. gondii* RH strain either ip or per os (intraduodenally). The levels of *T. gondii* DNA were assessed in blood, spleen, liver, lungs, heart, brain, eyes, lamina propria and Payer's patches at different time points post infection (PI) by RT-PCR. The recruitment of neutrophils, macrophages, dendritic cells, B cells and T cells to the peritoneum was studied by flow cytometry and the levels of cytokines (IFN-g, TNF-a, IL-2, IL-4, IL-6, IL-18) in spleen were assessed by PCR and flow cytometry.

Results: We found that the levels of toxoplasma DNA in orally infected mice were lower in the majority of the organs under the study (for example, spleen or liver) compared to ip infected mice. However they were higher in the brain and Payer's patches of orally infected mice. Earlier and more pronounced recruitment of inflammatory cells such as neutrophils, dendritic cells and macrophages to the peritoneum was observed in ip infected mice, while higher amounts of B cells (both $CD5^-$ and $CD5^+$) were found in orally infected mice starting from day 5 PI. Higher levels of inflammatory cytokines such as IL-1b, TNF-a and IL-18 were found in ip infected mice compared to per os infection.

Conclusions: Our results suggest that systemic toxoplasma infection directs the immune response towards better control of toxoplasma dissemination to the brain. Precise mechanisms of this control are currently under investigation.

P1186

NKT cell expansion in absence of B cells may be related to an increased regulatory NKT cell function during *Trypanosoma cruzi* infection

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Purpose/Objective: Chagas disease is a tropical disease caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*). We have previously demonstrated that Natural Killer (NK) cells were related to resistance to *T. cruzi* infection in C57Bl/6 mice. Depletion of total $NK1.1^+$ cells in C57Bl/6 infected mice resulted in diminished convertion of recently activated peripheral T cells in effector/memory lymphocytes, thus resulting in T cell hyperactivation without maturation to effector functions. Additionally, we have studied the role of B cells in the activation/differentiation of peripheral T cells during *T. cruzi* infection. We have found that muMT knockout (KO) mice were more susceptible to *T. cruzi* infection and had fewer central and effector memory T cells when compared with wild type mice. Taken together, these results might suggest a relationship between B cells and $NK1.1^+$ cells. The present study was designed to evaluate NKT cell-numbers in both muMT and WT infected mice.

Materials and methods: Mice were kept in microisolators and were manipulated according to institutional ethical guidelines. Spleen cells were isolated from C57Bl/6 and C57Bl/6 muMT KO mice (1–2 months old). B cells were obtained by magnetic separation and were adoptively transferred to muMT C57Bl/6. After infection with Tulahuen strain of *T. cruzi*, flow cytometry analysis of the spleens from muMT or from B-cell-sufficient C57Bl/6 mice were performed. Fluorochrome-conjugated anti-alpha beta and anti-NK1.1 monoclonal antibodies were used.

Results: During the acute phase of infection, NKT cells were decreased in B cell-transferred muMT, when compared to control muMT infected mice, suggesting that B cells may down-regulate NKT-cell numbers during the infection. As a result, decreased of effector/memory T cells and increased NKT cell number may be related to diminished capacity to mobilize inflammatory cells to infected tissues, resulting in the increase in parasite load.

Conclusions: NKT cell expansion in absence of B cells might be related to an increased negative regulatory NKT cell function latter on early chronic infection, helping to inhibit the generation of effector/memory T cells.

P1187

Nucleosides from phlebotomus papatasi salivary gland exacerbate leishmaniasis by generating tolerogenic dendritic cells-dependent treg

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Purpose/Objective: Phlebotomines saliva plays a crucial role in the establishment of *Leishmania* infection. Among several potent pharmacologic substances, we recently purified and identified adenosine-(ADO) and adenosine-monophosphate-(AMP) as saliva's active pharmacologic compounds presents on *P. papatasii* that inhibits dendritic cells-(DC) functions through PGE_2/IL-10-dependent mechanism. *AIM:* We evaluate whether ADO and 5′AMP are compounds present into *Phlebotomus papatasii* saliva responsible for leishmania establishment into vertebrate host and such immunomodulatory mechanism.
Materials and methods: C57BL/6WT or C57BL/6IL-$10^{-/-}$ mice were coinoculated with *L. amazonensis* promastigotes forms (1×10^5parasites/ear-i.d. route) in the presence of ADO+AMP or vehicle (PBS). The ear lesion size, parasites burden, cytokines production and inflammatory infiltrated were analyzed at 12nd week post infection.
Results: ADO+AMP mimicked the exacerbative effect of saliva in leishmaniasis, increasing parasites numbers and ear lesion. Enzymatic catabolism of salivary nucleosides reversed the SGE-induced immunosuppressive effect. Such effect was associated with pro-inflammatory cytokines reduction (IFN-γ, TNF-α) and IL-10 enhancement. Moreover, ADO+AMP failed to enhance ear lesion and parasite burden in IL-$10^{-/-}$ infected mice. Interestingly, nucleosides increased Tregs makers (GITR; CTLA-4; CD39; CD73 expression) on $CD4^+$ $CD25^-$ population, suggesting the induction of Tregs on T effector cells. Treg induction was associated with nucleosides-induced tolerogenic dendritic cells (tDC) expressing higher levels of COX_2 and IL-10. Furthermore, nucleosides-induced tDC displayed a semi-mature phenotype and produced lower levels of proinflammatory cytokines *in vitro* and *in vivo*.
Conclusions: We demonstrated that ADO and 5′AMP are constituents present in *P. papatasi* saliva that exacerbated leishmania infection. The exacerbative effect is associated with Tregs enrichment generated by nucleosides-induced tDC in the inflammatory foci.

P1188

Pharmaceutical sodium chlorite (DAC-N-055) acts as anti-parasitic and immunomodulating compound in the defense against *Leishmania* parasites

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Purpose/Objective: Cutaneous leishmaniasis, caused by *Leishmania* parasites, is characterized by chronic, frequently ulcerating and slowly healing skin lesions. An efficient local therapy is not available. The widespread use of intralesional injection of antimony is limited by emerging resistant parasites and high costs. In clinical trials we obtained evidence that local application of pharmaceutical sodium chlorite (DAC-N-055) after removal of necrotic tissue accelerated the wound healing. Based on these observations we investigated possible anti-parasitic, immunomodulating and wound healing effects of DAC-N-055.
Materials and methods: Anti-parasitic effects of DAC-N-055 on extra- and intracellular *Leishmania* were evaluated by microscopic and photometric analyses. The influence of DAC-N-055 on several mouse and human immune cells involved in skin wound healing [bone marrow-derived macrophages (BMM) and dendritic cells (BMDC), monocytes, fibroblasts, endothelial cells, keratinocytes, peripheral blood mononuclear cells (PBMC)] and their effector responses were investigated by mRNA (real-time PCR) and protein expression analyses (ELISA, Western blot, proteome array) as well as by an *in vitro* wound healing scratch assay.
Results: *In vitro* DAC-N-055 exerted a cytotoxic effect on extracellular *Leishmania* promastigotes, but did not influence intracellular *Leishmania* amastigotes in BMM. In IFN-gamma-stimulated BMM the expression of iNOS, production of leishmanicidal NO and release of TNF and IL-6 were enhanced by DAC-N-055. In BMDC and in BMM production of IFN-alpha/beta, which is required for the early iNOS expression in murine *L. major* infections, was increased by DAC-N-055 upon stimulation with *Leishmania*, IFN-gamma or LPS. DAC-N-055 also augmented the expression of TNF and IL-6 in human MonoMac6 monocytes and HaCaT keratinocytes. Moreover, it modulated the expression of several chemokines and cytokines in human PBMC. Unexpectedly, DAC-N-055 did not show effects on the proliferation and/or migration of fibroblasts, endothelial cells and keratinocytes in wound healing scratch assays.
Conclusions: Together, our data indicate that DAC-N-055 is a leishmanicidal and immunomodulating compound, which *in vitro* influences the production of several effector molecules and cytokines that are protective in leishmaniasis and involved in wound healing.

P1189

Proportions of CD_4^+ but not $CD8^+$ memory T cells are altered in older individuals chronically infected with Schistosoma haematobium

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Purpose/Objective: There are indications that the efficacy of vaccines can be reduced in helminth infected individuals but so far there are no

studies on how helminths may mediate this effect in human or experimental studies. The efficacy of vaccines relies on the effective generation and maintenance of $CD4^+$ and $CD8^+$ T cell memory immune responses. Therefore the aim of this study was to characterise human $CD4^+$ and $CD8^+$ memory T cell accumulation in people exposed to *Schistosoma haematobium* infection and relate this to host infection status and schistosome-specific immune responses before and after anti-helminthic treatment.

Materials and methods: Proportions of $CD4^+$ and $CD8^+$ memory T cells were analysed in a cohort of 105 participants who are lifelong residents in an area endemic for *Schistosoma haematobium*. Fifty-one participants were analysed following treatment with an anti-helminthic drug. In addition schistosome-specific effector antibody responses and the overall function of $CD4^+$ effector/memory cells were determined.

Results: Our results show that helminth infection is associated with alteration of $CD4^+$ memory T cell proportions. This alteration was not observed in $CD8^+$ memory T cells. The changes in $CD4^+$ memory T cells are associated with an impaired T_H1 response and changes in activation markers. Treatment with an anti-helminthic drug leads to alterations of the $CD4^+$ memory T cell pool. Furthermore, reduced $CD4^+$ memory T cell proportions were associated with lower schistosome-specific effector antibody responses.

Conclusions: In summary, individuals infected with *S. haematobium* who did not to develop protective immunity with accumulative exposure show alteration in the overall CD4 T cell memory. These findings have implications for the development of the natural or the vaccine induced immune response against schistosome-specific as well as against unrelated pathogens.

P1190

The effect of vaccination with recombinant *L. donovani* and *L. mexicana* gamma glutamyl cysteine synthetase by different routes of administration on host immune responses

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Purpose/Objective: Human leishmaniasis is a spectrum of diseases caused by protozoan parasites of the genus *Leishmania*. Drugs existing for the treatment of leishmaniasis have been unsatisfactory. Ideally a vaccine could prevent infection and would be a feasible control method as infected individuals are resistant to clinical re-infection. In this study the ability of recombinant *L. donovani* and *L. mexicana* gamma glutamyl cysteine synthetase (γGCS) to induce a protective immune response in BALB/c mice was determined.

Materials and methods: Animals were immunised by different routes with the recombinant proteins and the effect of vaccination on production of parasite-specific IgG1 and IgG2a was determined using an ELISA assay. In addition the effect of immunisation on cytokine production by *in vitro* stimulated splenocytes from immunised mice was determined.

Results: Immunization with γGCS inducing significantly higher titers ($P < 0.05$) comparing to control group.

Conclusions: Vaccination induced significant Th1 and Th2 immune responses after single dose treatment.

P1191

The expression of B blood group carbohydrate under control of FUT2 gene (19q13.3) increases the risk for digestive Chagas disease

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Purpose/Objective: The infection by *Trypanosoma cruzi* can result in distinct clinical manifestations of Chagas Disease (CD) including heart (cardiomyopathy) and gastro-intestinal disease (megaesophagus and megacolon). Different factors contribute for this disease but there are no genetic markers indicating what form of the disease will occur. Previous reports evaluated the ABO blood group carbohydrates in relation to CD but they did not explore the influence of *FUT2* gene which controls the expression of these carbohydrates in the gastro-intestinal tract. This study aimed to verify the combined effect of the *FUT2* gene and the ABO blood group carbohydrates in heart and gastro-intestinal CD.

Materials and methods: Two hundred and forty patients were enrolled (cardiomyopathy: $n = 120$; gastro-intestinal: $n = 120$). The *FUT2* genotyping and the ABO blood group carbohydrates were identified by PCR-RFLP and hemagglutination methods, respectively. The qui-square test, the exact Fisher's test, and the Odds Ratio were used to compare the proportions.

Results: The differences between heart and gastro-intestinal CD were not statistically significant in relation to the FUT2 gene (χ^2: 1.141; DF: 1; $P = 0.2854$) and the ABO blood group carbohydrates (χ^2: 1.855; DF: 3; $P = 0.6031$) when analysed in isolation. However, when these two markers were analysed in combination, the differences were statistically significant for gastro-intestinal CD (χ^2: 9.961; DF: 3; $P = 0.0189$) and associated with the expression B (B and AB phenotypes) carbohydrates (OR: 10.969; CI 95%: 1.415−85.022; $P = 0.0114$).

Conclusions: The role of the *FUT2* gene and the ABO blood group carbohydrates in CD remains unclear and there are no evidences that these carbohydrates act as receptors for *T. cruzi*. Since the ABO carbohydrates expressed in the gastro-intestinal tract under control of the *FUT2* gene are structurally distinct from those expressed in the heart tissues, maybe these differences increase the risk for CD in the gastro-intestinal tract, especially among those carrying the B carbohydrate. In conclusion the expression of B carbohydrate from ABO blood group system in the gastro-intestinal tract is associated with this form of CD.

P1192

The role of mast cells in protection against *Leishmania donovani* infection

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Purpose/Objective: Visceral leishmaniasis is a major health concern in many parts of the world and designing suitable interventions to control the disease relies on understanding the role of host immunity in controlling susceptibility/resistance to infection.

Materials and methods: The role of mast cells in *Leishmania donovani* infection was determined by comparing the outcome infection in *Kit (Wsh)* mast cell *deficient* mice and their wild-type counterparts.

Specific antibody responses and cytokine production by *in vitro* stimulated splenocytes from infected mice were assessed. The role of mast cells in neutrophil recruitment was determined by monitoring their levels during infection, studying the effect of depletion on the outcome of infection, and determining myeloperoxidase levels in tissues of WT and *Wsh* infected mice.

Results: Mast cell deficient mice had significantly lower liver and splenic parasite burdens compared to wild-type mice and this reduction was associated with significant inflammation in the spleen, based on spleen weight.

Conclusions: Results of studies showed that mast cells have an important role in controlling the outcome of infection and that neutrophils are involved in protection in both mouse strains.

P1193

Two unique IL-10 producing B cell subsets contribute to the host cytokine balance during Trypanosoma brucei infections

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Purpose/Objective: IL-10 producing B cells have been described as a new regulatory cell type important not only in balancing auto-immune diseases, but also in the establishment of tolerance during parasitic infections. Infection with *Trypanosoma brucei* elicits a strong pro-inflammatory response and although it has been established that the anti-inflammatory cytokine IL-10 is crucial to prevent death of the host from hyper-inflammation, the cellular source of IL-10 has not been clarified so far.

Materials and methods: Blood serum cytokine levels and cytokine production by total spleen and liver cells was measured by ELISA. The production of IL-10 by individual cells was assessed through intracellular flowcytometric staining for IL-10 and the use of GFP IL-10 transcriptional reporter mice.

Results: After control of the first parasitemia wave there is a peak of IL-10 production in the spleen. The majority of the IL-10 producing cells has a B cell phenotype and can be divided into a $B220^{int}CD19^{hi}TIM\text{-}1^{+}$ $CD23^{int}CD11b^{+}$ $CD5^{low}$and a $B220^{low}CD19^{int}$ $TIM\text{-}1^{-}$ $CD23^{-}$ $CD11b^{low}CD5^{-}$ B cell population. Both cells populations are IgM^{+} $CD21^{low}LY6C^{+}BST2^{+}$ $CD11c^{low}IgD^{low}CD1d^{-}$ $AA4.1^{-}$ $CD138^{-}$. Two IL-10 producing B cell populations, a $B220^{lo}CD19^{int}$ and a $B220^{int}CD19^{hi}$ can be found in the liver as well, exhibiting a similar phenotype as the splenic populations.

Conclusions: Using both intracellular staining for IL-10 and GFP IL-10 transcriptional reporter mice, our results suggest that two distinct regulatory B cell subsets with 'DC-like' features are the major producers of IL-10 in the spleen during *Trypanosoma brucei* infection and may be involved in activating regulatory immune responses.

P1194

Unravelling the events leading to inflammation-induced trypanosomiasis-associated acute anemia

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Purpose/Objective: African trypanosomes are extracellular parasites that cause infections in human and livestock in sub-Saharan Africa. The common hallmark of *T. brucei* infections is hyper-inflammation due to an ill-controlled immune reaction. During the acute phase of experimental murine trypanosomiasis, there is a 50% reduction in red blood cell counts. It has previously been shown that during several microbial infections in mice, acute anemia is caused by erythrophagocytosis, and IFNγ is the critical driver in this immunopathology. Our purpose is to unravel the mechanism(s) implicated in the acute inflammation-associated anemia observed following control of the first parasitemia peak.

Materials and methods: 8-week-old female C57Bl/6 mice were infected by intraperitoneal injection of 5000 *T. b. brucei* AnTat1.1E clone parasites. Parasitemia and red blood cell counts were performed at 2-day intervals using a hematocytometer. Lymphocyte cell populations were analyzed by flowcytometry.

Results: We previously observed that serum IFNg levels are highest during the first days post infection. Here, we report that $IFN\gamma^{-/-}$ mice display reduced acute anemia. Moreover, this reduced pathology is also observed in beta-2-microglobulin (β_2m) deficient mice but not in transporter associated with antigen processing 1 (TAP1) deficient mice, suggesting that the cell population which plays a major role in the induction of acute anemia is independent of TAP transport for surface expression of the relevant MHC I molecule. Future analysis of anemia profiles of $K^{b}D^{b\text{-}/\text{-}}$ mice will determine whether the cell population is dependent on MHC Ia or MHC Ib.

Flow cytometric analysis during the first days post infection reveals that at day 4 (right before the appearance of the anemic phenotype) there is a significant increase in the number and percentage of NKT cells, $CD8^{+}$ T cells and $NK1.1^{+}$ $CD8^{+}$ cells in the liver and blood of wild type C57Bl/6 mice.

Conclusions: Further flow cytometric experiments will determine the presence or absence of these cell populations in $TAP1^{-/-}$ versus $b_2m^{-/-}$ mice. In addition, it will be investigated which cell population is the major IFNg producer during the first days post infection.

Together, these data will help to identify the cell population(s) responsible for the acute anemia during the initial phase of *T. brucei* infection.

P1195

Vitamin E as an adjuvant in an Taenia crassiceps mouse immunization

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Purpose/Objective: In this study we evaluates the efficacy of vitamin E as an adjuvant, using *Taenia crassiceps* antigens against murine cysticercosis. The efficacy of vitamin E was compared with three well known experimental adjuvants (complete Freund's adjuvant, aluminum salts, and saponin), and assessed with respect to the humoral immune response elicited and the reduction in parasite load. Vitamin E is a well-known anti-oxidant and has been shown to exert immunostimulatory activity by dietary supplementation . The beneficial use of α-tocopherol formulated in an oil emulsion as an adjuvant has been utilized for the development of various veterinary vaccines .

Materials and methods: Groups of 5 BALB/c mice were immunized intramuscularly, subcutaneously, or intraperitoneally with different doses (10 μg/mouse) of *T. crassiceps* antigens emulsified in mineral oil emulsions that was prepared as previously described (Valdez *et al.*1994). Saponin (Sigma) was prepared at a concentration of 50 μg/mouse as reported previously (McColm *et al.*1982). Aluminum salts and vitamin E were prepared as described by Ito *et al.*, 2009 and Tengerdy and Lacerda.1991, respectively. The solution containing vitamin E and VF-Tcra was emulsified using (Ultrasonic Devices for Liquid Processing, 2 kw). Thirty days later, the mice were given a booster with the same immunizing dose of the same peptide in the same adjuvant as had been done before. Animals were bled from the retro-orbital plexus at 60 days after immunization. The sera were pooled and kept frozen in aliquots at -20°C.

Results: Anti-VF-Tcra IgG antibodies that were produced were detected by ELISA on day 60 in all groups independently of the immunization method and of the type of adjuvant used. The compartive study of the different adjuvants showed that vitamin E produced the high titre of antibodies and was a good adjuvant $P \leq 0.05$. SDS*PAGE shows the pattern of VF-Tcra and VF-tso. VF-tso pathogens presented various peptides of 8−158 kDa, and the VF-Tcra pathogen was rich in <20 kDa polypeptides. The protection was induced using VF-Tcra as an immunogen and the igG avidity Index was high with vitamin E as adjuvant.

Conclusions: In the present study, we evaluated the humoral immunity induced by vitamin E and VF-Tcra as a alternative vaccination and adjuvant strategies in a murine model of cysticercosis meanwhile further investigation are required to identifiy others aspects of the immune response.

Poster Session: Primary Immunodeficiency and Rare Diseases

P1196

A novel mutation in the WSXWS motif of the common gamma chain interleukin receptor in a patient with severe combined immunodeficiency

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Purpose/Objective: Molecular characterization of a primary immunodeficiency: severe combined immunodeficiencies (SCID) are a heterogeneous group of genetic inherited diseases characterized by a profound impairment of both cellular and humoral immune response. X-linked SCID (X-SCID) is the most frequent type, affecting almost half of the patients. X-SCID is characterized by a total absence or very low numbers of T cells and natural killer (NK) cells. B cells are present, although they are nonfunctional. A 9 month old boy was submitted to hospital for severe recurrent respiratory and gastrointestinal infections from his first month of life, severe malnutrition and failure to thrive. Laboratory studies showed lymphopenia (720 lymphocytes/μl), absence of T and NK lymphocytes, normal number of B cells and panhypogammaglobulinemia. Parents were not consanguineous, two brothers died at 5 and 7 months of age and he had three healthy sisters. An X-linked SCID was suspected. He developed pneumonia, muguet and systemic candidiasis. Adequate treatment and profilaxis against infections were prescribed in order to receive a transplant from one of his three HLA identical sisters.
Materials and methods: More than 200 different mutations have been identified in patients with X-SCID. Primers were designed to amplificate the eight exons of ILRG2 by polymerase chain reaction (PCR). The gene was sequenced by using an automatic sequencer (ABI PRISM), and the sequence was compared with the reference sequence of the gene.
Results: DNA sequencing in the patient revealed a T>G transversion at the nucleotide 713 (c.713T>G) resulting in the substitution of the serine in position 238 by a arginine: p.Ser238Arg.
Conclusions: X-SCID is caused by mutations in the IL2RG gene, coding for the cytokine receptor gammasubunit common to IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors, known as the common gamma chain (γC). Cytokine receptors of the γC group belong to the class I cytokine receptor family, which includes the receptors of other interleukins and hormones. They are heterodimeric or heterotrimeric transmembrane complexes expressed on the surface of cells that share a highly conserved Trp-Ser-X-Trp-Ser motif (WSXWS motif). This motif is essential in receptor function, acting as a molecular switch involved in receptor activation. The sequence variant found in the patient has not been previously reported, however, we consider it is a pathogenic mutation, as it affects the WSXWS motif.

P1198

Autoantibody specificity from T cell receptor alpha chain deficient mice

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Purpose/Objective: Mice deficient for the alpha chain of the T cell receptor (TCR $\alpha^{-/-}$ mice) spontaneously develop autoimmunity. Although these mice have a developmental defect of TCR α/β T cells, they are able to develop germinal center reactions (GCR) and produce all immunoglobulin (Ig) classes with help from TCR γ/δ cells and from a small population of TCR α^{-}/β^{+} T cells. Given the fact that GCR and antibody production is impaired in TCR $\beta^{-/-}$ mice, we believe that the small population of T cells with a receptor consisting of a pre-T α and a β chain are essential for GCR.
Materials and methods: In order to determine the age of disease onset, as well as, the specificity of the autoantibodies produced, we have followed TCR $\alpha^{-/-}$ mice for several months to check the presence, by immunofluorescence, of autoantibodies that recognize cellular structures.
Results: We found that, although at 3 months of age autoantibodies are still absent, at 5 months autoantibodies are present in significant amounts in the serum. Moreover, these autoantibodies have preferential specificity to nuclear cell components even though in some individuals anti-cytoplasmatic antibodies are also identified.
Conclusions: We, therefore, conclude that α/β T cell lymphopenia in TCR $\alpha^{-/-}$ mice is sufficient to drive GCR and antibody production, but with a bias towards auto-reactivity.

P1199

CD107a expression-based NK degranulation assays for classification of familial hemophagocytic lymphohistiocytosis (FHL)

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Purpose/Objective: HLH is an uncommon, serious disease, lethal whithout treatment, characterized by a fail on the granules dependent cytotoxic activity and therefore on the immune system regulation, manifests as an uncontrolled and fatal systemic inflammatory syndrome. NK cells degranulation assays constitute a recent laboratory tool that allows an early diagnosis of familial hemophagocytic lymphohistiocytosis (FHL). In order to validate this assay, we intended to study differences between healthy individuals (HC), FHL and secondary HLH patients and establish local references values of NK cells' CD107a expression.
Materials and methods: Within December 2011 and April 2012, 20 samples from suspected HLH patients and 24 HC samples were analyzed. NK lymphocytes were incubated in complete medium (resting NK cells) or IL-2 conditioned medium (IL-2/NK cells) and posteriorly co-cultured with K-562 cells. The quantification of the CD107a percentage expression on the NK lymphocytes surface (ΔCD107a%) and mean fluorescence intensity (MFI) were obtained by multi-parametric flow cytometry (BD Biosciences). Descriptive statistics, Mann−Whitney *U*-test and ROC curves analyses were performed.
Results: Genetic diagnosis of FHL was confirmed in 3/4 patients with abnormal degranulation activity through sequencing of *UNC13D*, *STX11*, *STXBP2* and *RAB27A* genes; while nine patients who fulfilled HLH criteria were diagnosed as secondary HLH. HLH diagnosis was dismissed in seven patients.

	HC (n = 24)	FHL (n = 4)	P	Secondary HLH (n = 9)	P
DCD107a% resting NK cells $X \pm$ SD (p5-p95)	32 ± 12 (13–55)	4.7 ± 3.8	0.002	28 ± 19	0.94
DCD107a% IL-2/NK cells $X \pm$ SD (p5-p95)	52 ± 13 (28–72)	18.8 ± 11.7	0.003	43 ± 24	0.49
MFI resting NK cells $X \pm$ SD (p5-p95)	89 ± 38.6 (43–191)	48 ± 14.7	0.015	108.6 ± 45.8	0.15
MFI IL-2/NK cells $X \pm$ SD (p5-p95)	134 ± 71.5 (48–324)	72.8 ± 54.8	0.057	177 ± 66.4	0.057

A resting NK cells DCD107a% cutoff value at 10.9 (p5:13%) was identified for classification of FHL forms.

Conclusions: Normal ΔCD107a% ranges, sensitivity and specificity are similar to those described by other authors. Therefore we confirm and validate the NK lymphocytes degranulation assays as a very useful technique for HLH immunologic diagnosis. Further studies are needed to assess whether higher values of MFI may be helpful for secondary HLH diagnosis.

P1200
Common variable immunodeficiency (CVID) and T immunoregulatory cells

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Purpose/Objective: CVID is a common immune defect in the adulthood. Besides the infective risk, CVID is characterized by an increased incidence of autoimmune diseases, mainly autoimmune cytopenias. Unbalance in the ratio between T regulatory cells (Treg, decreased) and Th17 cells (increased) has been reported in several autoimmune conditions. These cells represent targets for different immunomodulatory therapy, included intravenous immunoglobulin (IVIg). Both IVIg and subcutaneous immunoglobulin (SCIg) are currently used as Ig replacement therapy in CVID. In this study we decided to investigate the possible immunomodulatory role of SCIg therapy and, specifically, its impact on Treg and Th17 levels.
Materials and methods: Ten CVID patients were subdivided according to EUROclass classification and cytofluorimetric analysis of peripheral blood Treg and Th17 cells levels was performed before and after SCIg administration. As a comparation, 10 healthy volunteers were analyzed for Treg and Th17 cells levels.
Results: No significant correlation was demonstrated between B phenotype and T immunoregulatory cells levels. A significant reduction of Th17 level was observed 48–60 h after SCIG infusion (post versus pre: 1.12 ± 0.87% versus 1.71 ± 0.70%; $P < 0.05$). By contrast, no significant results were obtained comparing Treg levels before and after SCIg. As a result, Treg/Th17 ratio significantly increased after SCIg infusion (pre versus post: 1.60 ± 1.18 versus 2.68 ± 1.65; $P < 0.05$).
Conclusions: In CVID, SCIg seem not to play the only role of replacement therapy, since they significantly modified Treg/Th17 balance in our group of patients. This suggest the possibility of using SCIg as immunomodulatory treatment in different settings.

P1201
Disseminated BCG-infection in patient with autosomal recessive chronic granulematosis disease

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Purpose/Objective: Mycobacterial infection is not common cause of infectious syndrome in children with chronic granulomatosis disease (CGD).
Materials and methods: Observation of case with CGD with generalized BCG infection. Immunologic and genetic examinations were used for diagnosis of CGD.
Results: The child was born from III pregnancy, II delivery on 37th week of gestation with body mass 2750 g. She received BCG vaccination at 4th day of birth. At 3 months the local inflammation with regional lymphadenitis developed which was treated with isoniazid for 3 months. Then bilateral purulent cervical lymphadenitis developed at 6, 9 and 11 months treated with wide spectrum antibiotics. Culture from pus was negative. PCR for *Mycobacterium tuberculosis* complex was negative. Since 1 year old causeless periodic fevers occurred with progressive hepatosplenomegaly, loss of weight, progressive anemia, inflammatory changes in blood for almost 6 months. Bacteriological cultures were negative. Treatment with wide spectrum antibiotics had insufficient effect. Antitubercular treatment with amycacinum, rifampin and izoniazid was started *ex juvantibus* for 6 months and positive effect had been received. Three months after antitubercular treatment was stopped the child had pneumonia which did not give in to traditional treatment during 2 months. Then antitubercular treatment was renewed with positive effect. At the age of 7 during the unexplained fever multiple formations were identified in the liver, biopsy showed caseous mass, histologically confirming the mycobacterial nature of lesions. Immunological findings revealed normal serum CH_{50}, Ig A, IgM, IgG, IgE and lymphocyte subpopulations levels, NBT-test 2–14%, phagocytic activity 4–28%. At the age of five the blood samples were tested at INSERM and autosomal recessive CGD was confirmed ($p22^{phox}$).
Conclusions: Universal BCG vaccination of newborns may cause problems in patients with CGD. Difficulties in diagnostics of BCG infection require administration of anti-tuberculosis drugs in suspicious cases.

P1203
Enhanced formation of giant cells in common variable immunodeficiency

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Purpose/Objective: To examine the propensity of peripheral blood monocytes in Common Variable Immunodeficiency to fuse to form multinucleate giant cells in relation to the tendency to form granuloma.
Materials and methods: The fusion index of cells from individuals with Common Variable Immunodeficiency (CVID) and normal controls were compared in a range of cytokines, supernatants and mitogens over a week in culture. Cultured monocytes at different of differentiation were centrifuged onto coverslips and examined under electron and confocal microscopes.
Results: Cell fusion of CVID peripheral blood monocytes was variable but on average was quicker, approximately twofold more frequent and formed giant cells with higher numbers of nuclei in culture medium without cytokines than normal. Addition of IL4, GMCSF, IFNg, TNFa and T cell conditioned media further induced normal and particularly CVID giant cell formation and combinations of cytokines and

monokines acted synergistically in promoting monocyte fusion. Treatment with anti INFg antibody reduced normal giant cell formation particularly, indicating a greater predisposition of peripheral CVID cells to fuse, while a greater tendency of CVID cells to fuse with immunoglobulin conditioned media my indicate the contribution of IVIG treatment in granuloma formation. CVID and normal giant cells expressed similar levels of MHC class II and costimulatory molecules and FC receptors and demonstrated metabolic and phagocytic activity with bacteria, yeast and fluorescent carboxilated beads.

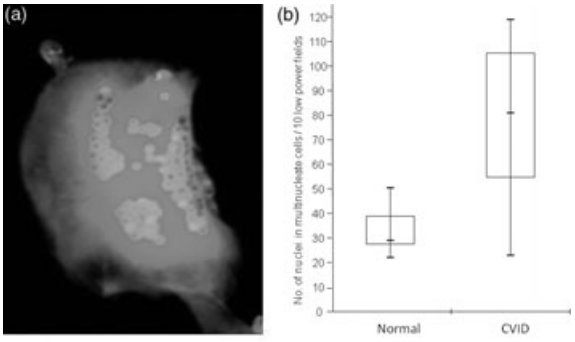

Figure 1: Appearance Of Multinucleate Cells In Culture. CVID mononuclear cells formed large multinucleate cells when cultured in GM CSF and IL4 (A): stained with MHC class II DR FITC and DAPI. (B): PBMC were plated in 6 well plates at a density of 5 x 10^5 cells per ml in GM CSF and IL4 and fused cells examined under a bright field microscope. Data displayed as A: mean (-), 25 to 75% (□) and complete range (⊥) of the fusion index of normal and CVID cells of 8 individuals.

Conclusions: A 2- to 5-fold greater tendency to form giant cells was induced in peripheral CVID monocytes by an extensive range of monokines, inflammatory lymphokines and T cell supernatants. CVID and normal cell giant cells were metabolically active and phenotypically similar.

P1204
Familial multiorgan-autoimmunity syndrome with cytopenia, liver-, and islet cell autoantibodies — a novel inherited immune dysregulation syndrome?

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Purpose/Objective: A now 16-years-old girl from a Kurdish consanguineous family underwent stem cell transplantation (SCT) from her HLA-identical mother for suspected type 3 (non-classic) autoimmune lymphoproliferative syndrome (ALPS) after suffering from chronic immune thrombocytopenia (ITP) starting from her 2nd year of life and recurrent bacterial infections, developing into generalized massive lymphadenopathy and splenomegaly without evidence of EBV infection. Soon after successful SCT lymphoproliferation resolved, but chronic ITP recurred. Both mother and daughter have normal immunoglobulin (Ig) levels and antibodies against viral and vaccine antigens, but were found to be positive for liver/mitochondrial autoantibodies (AMA, M2) without liver/biliary disease or cryptosporidia in stool.

A now 8-years-old sister was diagnosed with autoimmune hemolytic anemia (AIHA) at the age of five years. AIHA resolved under immunosuppression with rituximab, FK506 and MMF; total B cell numbers and Ig levels were normal prior to anti-CD20 therapy. Later, the girl developed a persistent CVID-like syndrome with low B cell numbers (<50−100 μl) and hypogammaglobulinemia, chronic enteropathy with growth arrest, positive tests for Noro-, Adenovirus, HSV, CMV. The girl is Ig-substituted, has hypothyroidism, anti-mitochondria- (AMA, M2), islet-cell, insulin- and glutamate decarboxylase- but no other autoantibodies. Family history including a 3-years-old healthy brother is unremarkable.

Materials and methods: Standard immune phenotypical assays including TCR-Vb-spectratying did not reveal a known type of cellular immunodeficiency. CD4/CD8 double-negative T cells were borderline increased (2−10%), but serologic parameters (VitB12, Apolipoprotein), apoptosis, killing, and lymphocyte proliferation assays were normal, lymph node histology from the older girl was 'compatible' with ALPS, but gene sequence of CD95, CD95L, Caspase-8, and -10 were normal. Bowel histology of the younger patient showed lymphocytic enteritis.

Results: In summary, one sibling shows an iatrogenous CVID-like syndrome and multiple viral infections after anti-CD20-treated AIHA, and the other girl partial remission of atypical ALPS post-HSCT.

Conclusions: In search for an unrecognized immune dysregulation syndrome, the currently ongoing steps of diagnostic work up are homozygosity mapping and exome sequencing.

P1205
Humoral immunity in patients with diGeorge syndrome

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Purpose/Objective: Syndrome diGeorge is a complex disorder caused by an embryopathy based on del22q11. Due to hypo/aplasia of a thymus resulting immunodeficiency affects mainly T cells. Recent data, however, show a pathology also in humoral immunity. We have, therefore, investigated B cell compartment and antibody response in a cohort of diGeorge patients.

Materials and methods: Total cohort of 50 patients was available for investigation. Immunoglobulin levels and antibody response against tetanus were measured repeatedly in all subjects, post-vaccination antibodies against Pneumococcus sp. and Hemophilus infl. in subcohorts (33 and 37, respectively). Seventeen patients, age 3 months till 20 years, were available for B cell phenotyping.

Results: Only 14% of patients had all immunoglobulin isotypes in normal range. We have observed higher tendency to increased than decreased values of immunoglobulins (increased IgG 24%, IgA 33%, IgM 5%, decreased IgG 12%, IgA 10%, IgM 17%). Seventy two percent of patients had normal post-vaccination response, all others presented with low values of specific antibodies. All tested patients showed markedly increased population of transitional and naïve B cells (14/17 samples above median) and decreased switched memory B cells (17/17 samples below median). Decrease of switched memory B cell is highly significant and progressive with age.

Conclusions: B cell compartment and antibody response are disturbed in diGeorge patients. Regular investigation of humoral immunity would help in clinical management and vaccination strategy.

P1206

IL-12/23-IFN-gamma axis defects and their effects on human NADPH oxidase system activation – myelomonocytic cell model

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Purpose/Objective: To evaluate the NADPH oxidase in myelomonocytic human cells with IL-12/23-IFN-γ axis defects.
Materials and methods: Cell model: wild type U937 cells or treated with control, IFN-γRα (IFNGR1) or IFN-γRβ (IFNGR2) shRNA lentiviral particles for the silencing of IFNGR1 or IFNGR2 gene. The cells were differentiated or not with PMA or IFN-γ+TNF-alpha before the experiments. NADPH oxidase activation was measured by flow cytometric assay using dihydrorhodamine 123 (DHR); CYBB, CYBA, NCF1 and NCF2 gene expression was evaluated by real-time PCR; phagocytic activity was measured by cytometric assay using Paracoccidioidomicose brasiliensis marked particles. We also analysed the flavocytochrome b558 and CD54 (ICAM-1) expression by flow cytometric assay.
Results: U937 shRNA IFNGR1 and U937 shRNA IFNGR2 lineages showed impairment of NADPH oxidase activation measured by DHR assay when stimulated with IFN-γ, PMA or IFN-γ+PMA. There was a decrease on flavocytochrome b558 Median Fluorescence Intensity (MFI) in the IFN-γ-stimulated U937 shRNA IFNGR2 lineage. The CD54 MFI was found diminished mainly in IFN-γ-stimulated U937 shRNA IFNGR1 lineage. The gene expression of the components of NADPH oxidase system evaluated here was decreased in U937 shRNA IFNGR1 and U937 shRNA IFNGR2 lineages, stimulated or not with IFN-γ. The silencing of IFNGR1 or IFNGR2 gene did not influenced the phagocytic capacity of IFNGR1 or IFNGR2 deficient cell lineages.
Conclusions: The activation defect of NADPH oxidase system showed in our myelomonocytic human cell model can contributes to explain the susceptibility to Mycobacteria in Mendelian Susceptibility to Mycobacterial Diseases as well as the reduction of ICAM-1 showed here.

P1207

Immunoglobulin G fraction of APECED sera inhibits type I interferons and Th17-related interleukins

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Purpose/Objective: Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) is a recessive disorder resulting from mutations in the autoimmune regulator (*AIRE*). In addition to organ-specific autoantibodies, almost all of these patients have serum autoantibodies to several cytokines. Those against type I IFNs are specific and sensitive disease markers. Autoantibodies to Th17-related interleukins are associated with chronic mucocutaneous candidiasis. APECED sera have high neutralizing capacities against the respective cytokine. Although these are assumed to be caused by serum autoantibodies it has not yet been proved directly.
Materials and methods: In this study, cell-based cytokine neutralization tests were performed with purified IgGs and IgAs from APECED patients' and control sera to test serum immunoglobulin neutralization capacity. In addition, luciferase immunoprecipitation systems (LIPS) assays were carried out to specify the dominant IgG subclass(es) in APECED and thymoma patients against these cytokines and to find out the main immunogenic epitopes of IFN-α2a and IL-22.
Results: Neutralization of cytokine function was detectable only with APECED IgG and IgA samples. IFN-ω and IFN-α2a were neutralized by IgG with significantly higher efficiency than by IgA, while IL-22 and IL-17A were neutralized almost exclusively by IgG. Their dominant isotypes proved to be IgG1 and surprisingly IgG4 (but not IgE), thus implicating Th2 or regulatory T-cell responses in their initiation. Patients with thymomas showed striking parallels, possibly implicating epithelial autoimmunization in these AIRE-deficiency states. The epitopes on IL-22 and IFN-α2a appeared to be mainly conformational, but some mapped closer to their C-termini.
Conclusions: In conclusion, the predominance of IgG type anti-cytokine autoantibodies over IgA suggests that autoimmunization against cytokines in APECED is not just secondary to chronic *Candida* infection at mucosal surfaces but intimately related to the disease pathogenesis.

P1209

Somatic mutations in immunodeficiencies: is it a frequent event in '*de novo*' PID?: suggestions from a case report of X-linked Severe Combined Immunodeficiency

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Purpose/Objective: X-linked Severe Combined Immunodeficiency (X-SCID) is due to deleterious IL2RG mutations, and represents the most frequent type among SCIDs. The healthy mother may carry a mutated chromosome and transmit it to the offspring. Alternatively, a *de novo* occurring mutation in the patient can occur. Rarely in this disease, somatic mutations have been described. To define the pattern of inheritance (*de novo* or maternally inherited) of an already known p.R226C IL2RG mutation in a classical X-SCID patient.
Materials and methods: Sanger-based sequencing of gDNA of the mother and her affected son. Confirmation of the mosaicism by high throughput sequencing (with high coverage level).
Results: By Sanger-based sequencing methods, we detected in patient's mother peripheral blood the nucleotide exchange as a potential somatic mutation, with low-level mosaicism that was confirmed by massive parallel sequencing with high coverage. The vertical transmission of the deleterious IL2RG allele to her male offspring indicated its presence in gonadal tissues. The mutation was absent in maternal patient's grandparents. Altogether, these evidences support that a *de novo*, somatic IL2RG mutation occurred in this carrying mother, most probably early during embryogenesis.
Conclusions: The present report describes a rare and unusual genetic phenomenon, emphasizing the inclusion of X-SCID in the group of genetic diseases in which somatic mutations can play a role in the pathogenesis. Since they can be vertically transmitted, their identification has serious implications in genetic counselling. However, their detection is usually difficult by conventional Sanger-based sequencing methods. Thus, similar studies should benefit of the systematic use of sensitive and quantitative genetic methods.

P1210

Study of a functional c-kit rs6554199 polymorphism and achalasia in a Spanish population

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Purpose/Objective: Idiopathic achalasia, a primary motility disorder of the esophagus characterized by failure of the lower esophageal sphincter (LES) and aperistalsis, is mainly caused by loss of the inhibitory effect of the esophageal myenteric plexus and subsequent LES relaxation. The possible etiologies proposed for achalasia include neuronal degeneration, viral infection, genetic predisposition and immune-mediated destruction of the myenteric plexus. The *c-kit* gene encodes a tyrosine kinase receptor expressed among others by interstitial cells of Cajal (ICCs), which together with the enteric nervous system are involved in gastrointestinal motility. In fact, alterations in ICCs distribution have been reported in different intestinal disorders. A functional polymorphism (rs6554199) located in the *c-kit* gene has been reported associated with achalasia in a Turkish cohort. Our aim was to replicate the aforementioned result in a large cohort of Spanish patients and controls.
Materials and methods: A case control study was performed with 282 Spanish white unrelated patients diagnosed of achalasia and 687 healthy controls. All of them were genotyped for the SNP rs6554199 using a TaqMan Assay by Design (Applied Biosystem, Foster City, CA, USA) analyzed in a 7900HT Fast Real-Time PCR System.
Results: No association was found for the *c-kit* rs6554199 polymorphism and achalasia in our study. The frequency of the T allele was similar in patients and controls (47.3% versus 49.4%; OR = 0.92, $P = 0.41$) and no trend for association was detected in genotypes or carriers of T allele. Additionally a gender stratified analysis was performed and again no differences were found.
Conclusions: The finding that the T allele of the *c-kit* rs6554199 polymorphism could be associated with achalasia, as previously reported in a Turkish population could not be replicated in a Spanish cohort. Although ethnic differences might explain these data, the sample size that compromised the statistical power in the Turkish cohort and is higher in our study, led us to conclude that the reported association seems to be a false positive.

P1211

Study of gamma delta T cells in a Tab-Tgd+B+NK+ SCID due to CD3D mutations

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Purpose/Objective: CD3δ deficiency is usually associated to absent T lymphocytes. However, a leaky CD3D mutation leading to half CD3δ protein levels in homozygous SCID patients allowed for normal numbers of peripheral blood $\gamma\delta$ T cells. Our objective was to study the phenotype, function and V region usage of $\gamma\delta$ T cells from two unrelated infants *ex vivo* and *in vitro*.
Materials and methods: Flow cytometry, cell culture and TCRD V01−3 spectratyping.
Results: Despite their normal numbers, $\gamma\delta$ T cells showed a stronger reduction of surface TCR expression levels (5-fold) as compared to $\alpha\beta$ T cells (2-fold) in both patients. Upon CD3 triggering, both TCR isotypes modulated well but signalled poorly. TCRD V usage by $\gamma\delta$ T cells was, however, quite normal as judged by spectratyping and flow cytometry, and was not perturbed by CMV infection, which was ascertained in one of the patients. The CD4- $\gamma\delta$ T cell subset (both DN and CD8+), was normally represented. In contrast, the CD4+ $\gamma\delta$ T cell subset, which is marginal in healthy individuals (<2%), was strongly expanded in both patients (30%), had an activated phenotype and could be grown *in vitro* with feeder cells. Normal CD4+ $\gamma\delta$ T cells are frequent in fetal liver, poorly cytolytic compared to CD4- $\gamma\delta$ T cells, but good MHC class II- and CD4-independent B cell helpers.
Conclusions: The results indicate that low CD3δ strongly impairs $\gamma\delta$ TCR expression but not $\gamma\delta$ T cell selection or diversity, particularly of the rare CD4+ $\gamma\delta$ T cell subset.

P1212

The presence of intermediate phenotype of CD4 T cells in CVID is a result of increased CD70 expression on activated T and B cells

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Purpose/Objective: Common Variable Immunodeficiency (CVID) is characterized by low levels of serum IgG, IgA, and/or IgM and impaired specific antibody response after antigen challenge (Conley Clin Immunol 1999). Investigation of peripheral blood (PB) cellular compartments revealed abnormalities in B and T cells (Vlkova Clin Exp Immunol 2006). Using polychromatic flow cytometry and probability binning computational approach (Kalina Cytometry A 2009) we found accumulation of CD4pos T cells with increased proportion of intermediate (Appay Semin Immunol 2004) CD28posCD27neg cells in CVID patients. We asked if the disappearance of CD27 on CD4pos T cells could be related to prolonged expression of its ligand CD70 on B and T cells or dendritic cells (DCs). Since CD70 is expressed transiently upon activation on B and T cells (Arens Immunity 2001) we analyzed activation status of immune system of CVID patients based on CD69 and changes in cytokine milieu in PB.
Materials and methods: We analysed expression of CD70 and CD69 on PB B cells, T cells and DCs and monitored 11 plasma cytokines of CVID patients ($n = 21$) and healthy age-matched controls ($n = 18$) using 8-color flow cytometry.
Results: Expression of activation marker CD69 was more frequent on B andT cells of CVID patients compared to healthy controls (mean 11.1% versus 1.5% on B cells and 7.6% versus 1.7% on T cells, $P < 0.05$). CD70 was more frequent on naïve CD27neg B cells (8.6% versus 2.6%), less frequent on mature CD27pos B cells (11.8% versus 34.7%) and more frequent on CD3pos T cells (6.8% versus 1.5%) [both CD4pos (8.9% versus 1.6%) and CD8pos (6.3% versus 1.5%)] in CVID patients compared to healthy controls ($P < 0.05$). Neither CD69 nor CD70 were expressed on DCs. Surprisingly, we did not observe elevated expression of cytokines usually connected with immune system activation such as IFNg, IL-2, IL-4, IL-5 or IL-6 in plasma of CVID patients.
Conclusions: Since CVID pathogenesis is clearly heterogeneous, it is difficult to pinpoint the cause versus the effect of the deregulation in T to B cell interaction. Here we show that accumulation of CD4pos T cells with intermediate CD28posCD27neg phenotype could be explained by engagement of CD27 by its ligand CD70 expressed on activated B and T cells. This is not accompanied by changes in inflammatory cytokines levels. Our findings add evidence to the B × T cell interplay defect in CVID patients.

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P1213

The role of LEKTI deficiency in immunity and skin homeostasis

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Purpose/Objective: Comël-Netherton syndrome is a rare autosomal recessive disorder characterized by ichthyosis, bamboo hair, atopic diathesis, recurrent infections, and a 20% fatality rate in the first year of life. Mutations in *SPINK5* lead to reduced expression or complete absence of the protein it encodes the serine protease inhibitor LEKTI. In 2009 we defined the Comël-Netherton syndrome as a primary immunodeficiency disorder. This fact drew our attention to LEKTI's role in immune defense and to its potential effect on one of the most important serine proteases granzyme B (GZMB). LEKTI is expressed in epithelial cells but not in leukocytes. LEKTI fragments, however, have been isolated from human blood.

Materials and methods: We investigated the influence of the full length LEKTI protein and its various fragments (domains 1−6, 6−9, 9−12 and 12−15) on GZMB activity using an enzymatic assay and performed flow cytometric analysis using Annexin V-7AAD co-staining to examine a possible apoptotic effect by LEKTI.

Results: High amounts of LEKTI protein resulted in increased GZMB activity while low concentrations showed a mild reduction of GZMB activity suggesting a direct influence of LEKTI on GZMB. Since GZMB is an important player in T cell and NK cell mediated apoptosis we investigated a possible role of LEKTI on GZMB mediated cell death. Our experiments so far suggest a dose-dependent influence on GZMB mediated apoptosis by LEKTI.

Conclusions: We conclude that LEKTI secreted by epithelial cells may contribute to the induction of apoptosis in skin infiltrating T cells preventing unlimited cytotoxicity by these skin infiltrating cells against epidermal cells and hence play an important role in the maintenance of homeostasis in inflammatory skin processes. Thus the defective regulation of apoptosis may explain the increased incidence of recurrent infections, severe allergic manifestations and skin inflammation characteristic for the Comël-Netherton syndrome.

P1214

The Spain national registry of primary immunodeficiency diseases

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Purpose/Objective: Primary immunodeficiency diseases (PIDs) are acknowledged as important medical and social issues. Registries of PIDs are essential for improving our knowledge of many features of these rare disorders. The Spanish Registry for Primary Immunodeficiency Diseases (REDIP) was established in 1993. The online open-access database version was launched in 2005 and since 2009 REDIP contributes to the European Society for Immunodeficiencies (ESID) Registry.

Materials and methods: Demographic and clinical data of PID patients were registered by physicians from all over Spain. Collected data is anonymous and in agreement with ethic and protection guidelines.

Results: REDIP nowadays comprises 1890 patients from 27 regional centres located in 11 autonomous communities, with an overall prevalence of 4 PIDs per 100.000 inhabitants. Selective immunoglobulin IgA deficiency represents the most common entity (n = 722; 38.3%), followed by common variable immunodeficiency (n = 400; 21%), C1-inhibitor deficiency (n = 234; 12.4%), IgG subclass deficiency (n = 87; 4.6%), X-linked agammaglobulinemia (n = 61; 3.2%) and DiGeorge anomaly (n = 51; 2.7%). Consanguinity and familial antecedents were reported in 3.4% and 23.7% of cases, respectively. Autoimmunity was the most frequent complication (248; 13%) and celiac disease the most prevalent condition (41; 16.5% of total autoimmune disorders). The most frequent long-term therapy was immunoglobulin replacement (629; 33% of patients).

Conclusions: Overall prevalence of PIDs in Spain is similar to that observed in other national registries. However, the prevalence of Complement deficiency is higher than others registries, related to the incorporation of National C1inhibitor Registry to REDIP. Registries are powerful tools to understand different aspects of PIDs and promoting collaboration among healthcare professionals.

P1215

Why don't to do severe combined immunodeficiency newborn screening in Europe?

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Purpose/Objective: Infants affected by Severe combined immunodeficiency (SCID) die of infectious complications in the first years of life, unless an early diagnosis and a hematopoietic stem cell transplantation (HSCT) have been performed. They are not often diagnosed promptly. The delay in diagnosis and treatment has awful consequences. SCID infants receiving HSCT before 2 months of age show 95% of survival, whereas those receiving HSCT after 5 months show 70% of survival and 30% after 8 months. Early diagnosis is not always easy, and many of these patients die without being diagnosed.

Lymphopenia is the most constant sign of SCID. Its detection by T cell receptor excision circles quantification (TRECs) is the used method for screening.

The USA experience on SCID Newborn screening have been successful. We propose and discuss the convenience of introduce it in Europe based on clinical benefits and cost-effectiveness.

Materials and methods: A systematic literature review of published report was performed using the internet database Pubmed. Search criteria were; 'severe combined immunodeficiency', 'neonatal screening', 'T cell receptor excision circles' 'Hematopoietic stem cell transplantation', 'cost-effectiveness analysis' and 'QALY'. All retrieved papers were screened for additional references.

Results: Sixty three papers were analyzed and the main findings were:

Screening for SCID began in Wisconsin and Massachusetts in 2009. Following the recommendation of the USSHHS, California, Louisiana, New-York, Florida and Puerto Rico have implemented screening programs from 2010 to 2011 and, it has been extended to all states in 2012.

Main clinical results: of the widest study, carried out in California: 10 SCID has been detected among 507.000 newborns (two γc chain deficiency, two IL7Rβ deficiency, two RAG1 deficiency, one omenn syndrome and three SCID variants). Implementation of the program allowed to get 880 life years. Cost-effectiveness: the program show an incremental cost-effectiveness ratio of 25.429 $/life year, and of 27 907$/Quality adjusted life year (QALY), and allowed to get 802 QALYs.

Conclusions: American experience on SCID screening program has shown clinical benefits and suitable cost-effectiveness balance. Why Europe countries appear not to be so interested in it?

Poster Session: Rheumatoid Arthritis and Lupus

P1216

11ß-hydroxysteroid dehydrogenase-1 amplification of glucocorticoid action in myeloid cells promotes resolution of K/BxN arthritis in mice

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Purpose/Objective: Glucocorticoids (GCs) have been widely used to treat arthritis. However, despite the efficacy of synthetic GCs, the role played by endogenous GCs in arthritis remains obscure. *In vivo*, endogenous GCs exist in active (cortisol and corticosterone) and inactive (cortisone and 11-dehydrocorticosterone) forms, the latter with negligible affinity for receptors. 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) converts inactive GCs into active GC, thereby amplifying GCs action within cells, *in vivo*. Macrophages express 11β-HSD1 and mice deficient in 11β-HSD1 (*Hsd11b1*$^{-/-}$) show delayed acquisition of macrophage phagocytosis. Compared to wild-type (WT) controls, *Hsd11b1*$^{-/-}$ mice show an earlier onset of inflammation in the K/BxN serum transfer model of arthritis, which is dependent on myeloid cells. *Hsd11b1*$^{-/-}$ mice also show delayed resolution of K/BxN arthritis, with greater exostosis of the bone. To dissect contributions of 11β-HSD1 in myeloid cells to this inflammatory phenotype, we have crossed *LysM-cre* with *Hsd11b1*$^{flox/flox}$ mice to generate *LysM-Cre Hsd11b1*$^{flox/flox}$ (MKO) mice.
Materials and methods: MKO mice show ~82% reduction of 11β-HSD1 enzyme activity in peritoneal resident macrophages, but little or no reduction in thioglycollate elicited macrophages. To induce arthritis, MKO and control *Hsd11b1*$^{flox/flox}$ and *Hsd11b1*$^{-/-}$ mice (male, aged 7–10 weeks, $n = 6–7$) were injected with K/BxN serum (125 μl/mouse). Clinical scoring of the joints was carried out blind to genotype for 21 days.
Results: Whereas *Hsd11b1*$^{-/-}$ mice showed an earlier onset of arthritis, as previously described, there was no difference in the onset or peak phrases of arthritis (day 1–12) in MKO mice compared to WT. However, inflammation was significantly worse in MKO mice between day 13–18, similar to *Hsd11b1*$^{-/-}$ mice.
Conclusions: These data demonstrate that resident macrophage 11β-HSD1 promotes the resolution of inflammation in K/BxN serum induced arthritis, but plays no role in the initial worse inflammation seen with global 11β-HSD1 deficiency in this model. In the future it will be important to elucidate the mechanisms underlying the pro-resolution role for resident macrophage 11β-HSD1, including whether its role in promoting macrophage phagocytosis of apoptotic cells is important in rheumatoid arthritis.

P1217

A novel method for investigating macrophage phagocytosis in juvenile systemic lupus erythematosus using the pH sensitive dye pHRodo

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Purpose/Objective: Phagocytosis is the regulated cellular uptake of pathogens, cellular debris, and apoptotic cells in a non-inflammatory manner. There is increasing evidence that systemic lupus erythematosus (SLE) autoimmunity could be related to impaired or delayed clearance of apoptotic cells since key autoantigens are found on the surface of apoptotic cells [1]. Studies have also shown macrophages derived from SLE patients to have impaired phagocytic ability [2]. Juvenile-onset SLE (JSLE) is a more severe form of adult-onset SLE and at present there are no studies into the role of phagocytosis within JSLE. The use of pHRodo is a novel method of investigating phagocytosis as it is a pH sensitive dye with a low fluorescent signal at the neutral pH but will emit a red fluorescence in the acidic environment of the phago-lysosome once it is engulfed by the phagoctye.
Materials and methods: CD14$^+$ cells were isolated from healthy control patients and used immediately or differentiated into macrophages in the presence of M-CSF. Following optimisation, macrophages from 3 donors and monocytes from 1 donor were incubated with 50 μl pHRodo stained *E. coli* in the presence of 10% JSLE [($n = 5$ macrophages) ($n = 3$ monocytes)] or control serum [($n = 4$ macrophages) ($n = 4$ monocytes)] for 30 min at 37°C (phagocytosis assay) or at 4°C (negative control). The cells were washed and analysed on a flow cytometer. The percentage of *E. coli* phagocytosis was determined by measuring the number of macrophages or monocytes emitting red fluorescence. Non-specific background fluorescence was removed by subtracting the percentage of phagocytosis in the 4°C negative control from the 37°C phagocytosis assay.
Results: Macrophages had a lower level of phagocytosis when incubated in JSLE serum compared with control serum (33.8 ± 5.0% versus 42.0 ± 7%). Monocytes had a lower level of phagocytosis in JSLE serum compared to control (33.0 ± 8.9% versus 42.7 ± 6.2%), although these differences were not statistically significant.
Conclusions: These results correspond with literature from adult-onset SLE patients suggesting that the serum environment within JSLE patients is able to inhibit effective macrophage phagocytic uptake. Further work analysing JSLE monocyte and macrophage phagocytosis rate and their ability to phagocyte apoptotic cells is underway.
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P1218

A role for the autoantigen La in the regulation of IFN production and progression of SLE?

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Purpose/Objective: SLE is characterised by chronic inflammation driven by immune complexes against autoantigens and overproduction of inflammatory cytokines and type I IFNs. La is a 48 kDa RNA-binding protein well documented as an autoantigen in SLE, with a known role in binding and stabilising nascent RNA polymerase III (RNA pol III) transcripts. Recent studies have shown that RNA pol III transcribes dsDNA into 5'ppp-dsRNA, a potent activator of cytosolic receptor, RIG-I, thus contributing to the production of type I IFNs in an antiviral response. Due to this recently uncovered role for RNA pol III, as well as the role of La in binding RNA pol III transcripts, we hypothesise that La may play an important role in regulating IFN production.
Materials and methods: Luciferase assays.

HeLacells were transiently transfected with a luciferase reporter construct containing the IFN-β p125 promoter (40 ng). TK Renilla (40 ng), and 50 ng of RIG-I, IPS-1, TRIF and TBK-1, Cells were assayed for reporter gene activity 18 h post-transfection.

Interaction studies.

Immunoprecipitation studies in HEK293T cells were carried out using Protein G sepharose beads pre-coupled to either monoclonal La. Pulldown was carried out using nickel agarose beads coupled to either full-length or truncated recombinant La. HEK293T lysates overexpressing RIG-I domains were added to these beads. All samples were analysed by western blotting.

Results: Our results indicate a novel role for La in directly and specifically inhibiting RIG-I-dependent IFN production. Transient transfection of HeLa cells with increasing amounts of La inhibits both RIG-I- and IPS-1- induced IFN-β promoter activity, whilst IFN-β promoter activity driven by TRIF, an adaptor downstream of TLR3 and TLR4, or the downstream effector kinase TBK-1 was unaffected. Co-immunoprecipitation and pulldown experiments demonstrated that La and RIG-I interacted via the N-terminal domain of La directly and the CARD domain of RIG-I, suggesting that the inhibitory effect of La on IFN-β activity is due to direct binding.
Conclusions: This work demonstrates that La is a novel negative regulator of IFN responses and may be important in protecting cells from recognising and responding inappropriately to self-dsRNA motifs that may potentially activate RIG-I. Future work aims to characterise the RIG-I/La interaction in SLE patients in order to establish the potential influence of this complex within the context of disease.

P1220
Angiogenic T cells and endothelial progenitor cells depletion in rheumatoid arthritis patients

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Purpose/Objective: To study the Angiogenic T Cells (Tang) in rheumatoid arthritis patients and their relationships with Endothelial Progenitor Cells (EPC), clinical and immunological parameters and traditional cardiovascular risk factors.
Materials and methods: Tang, defined as $CD3^+$ $CD31^+$ $CD184^+$ cells, were quantified in peripheral blood samples from 35 patients with long-standing RA and 17 healthy controls (HC) and related to EPC population, determined by flow cytometry based on CD34, VEGFR-2 and CD133 markers. Clinical and immunological data and traditional cardiovascular risk factors were obtained by reviewing clinical records. DAS28 score was used for measuring disease activity.
Results: RA patients showed significant Tang and EPC depletions compared with HC ($P < 0.001$, in both cases). As expected, both populations were positively correlated in HC ($r = 0.833$, $P < 0.001$) and inversely related to traditional cardiovascular risk factors. In RA patients, however, these associations were missing but Tang cells were negatively correlated with disease activity (DAS28: $r = -0.552$, $P = 0.003$). Moreover, RF-, anti-CCP- and ANA-positive patients exhibited a higher Tang depletion than their negative counterparts ($P = 0.006$, $P = 0.098$ and $P = 0.001$, respectively), thus linking disease-specific autoantibodies with lower endothelial-repair ability.
Conclusions: Tang and EPC depletion could have a role in determining the increased endothelial damage in RA patients. Particularly, Tang depletion could be impairing EPC functionality in RA patients. Additionally, autoantibody profile could be an interesting tool to determine specific-patient cardiovascular risks.

P1222
Anti-carbamylated protein antibodies are present in mice with collagen induced arthritis

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Purpose/Objective: Antibodies against citrullinated proteins (ACPA) are a characteristic of rheumatoid arthritis (RA). Carbamylation is a different type of post translational modification, where a Lysine amino acid is converted into a homocitrullin. Recently, we identified antibodies binding to carbamylated proteins (anti-CarP) in a subgroup of RA patients. In ACPA negative RA patients anti-CarP antibodies associate with joint damage. The aim of this study was to determine whether these anti-CarP antibodies are present in animal models of arthritis.
Materials and methods: Collagen induced arthritis (CIA) was induced in DBA/1 ($n = 28$) and C57Bl/6 ($n = 19$) mice by immunization with type II collagen in CFA. Arthritis severity was monitored using a clinical scoring system. Non-immunized animals ($n = 9$) served as negative controls. After disease onset serum was collected and antibody levels were determined by ELISA. The specificity of our anti-CarP ELISA was validated using dotblots.
Results: Whereas no anti-CarP antibodies could be detected in non-immunized DBA/1 mice, anti-CarP total Ig was present in 93% of the arthritic DBA/1 mice. Of those mice 36% had IgG1 and 79% had IgG2a anti-CarP antibodies. Antibodies to citrullinated proteins could not be detected. The levels of mouse collagen-specific IgG2a correlated with the clinical score. However, the levels of the different anti-CarP isotypes did not. Around 60% of the immunized C56Bl/6 mice developed arthritis. Anti-CarP IgG2c could be detected in 55% of those mice and could not be detected in the mice that did not develop CIA. Anti-CarP IgG1 was detected in 27% of the arthritic mice. Interestingly, mouse collagen specific IgG2c antibodies were detected in 100% of the immunized C57Bl/6 mice. Dotblot analysis, using carbamylated and non-modified proteins confirmed the ELISA results regarding the specificity of the antibodies for homocitrulline containing proteins.
Conclusions: Mice with CIA have antibodies to carbamylated proteins and their presence associated with disease development. All immunized mice have anti-mouse CII antibodies, indicating that the presence of anti-CarP antibodies could be a disease specific marker for arthritis in mice. Further studies will be required to determine the role of anti-CarP in the pathogenesis of arthritis.

P1223
Anti-inflammatory effects of phosphodiesterase 4 inhibitor roflumilast in experimental arthritis

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Purpose/Objective: Inhibitors of cyclic AMP (cAMP)-specific phosphodiesterase (PDE) are potential therpeutic agents with board anti-inflammatory and immuno-modulatory activities. PDE4 is the predominant family of PDEs expressed in a variety of inflammatory cells, and implicated in a number of immune responses including pro-inflammatory cytokine TNFα production.
Materials and methods: Using roflumalist, a potent and selective PDE4 inhibitor recently launched for the treatment of chronic obstructive pulmonary disease (COPD), we studied the therapeutic potential and immuno-modulatory effects of PDE4 inhibition in murine collagen-induced arthritis (CIA).
Results: Roflumilast significantly reduced the levels of TNFα in lipopolysaccharide (LPS) – primed BALB/c mice *in vivo* and RAW 264.7 macrophages *in vitro*. In a murine CIA model, oral administration of roflumilast (5 mg/kg) significantly inhibited disease severity, reduced articular inflammation and joint destruction. Treatment of roflumilast also reduced pro-inflammatory cytokine IL-6, TNFα, and IFNγ production in response to type II collagen *in vitro* and down-regulated serum levels of IgG1 and IgG2a anti-collagen antibodies. Moreover, marked reduction in disease activity and articular destruction was observed in mice treated with a combination of suboptimal doses of roflumilast and methotrexate (MTX; 1 mg/kg respectively).

Conclusions: These data demonstrate PDE4-sensitive inflammatory pathways in experimental arthritis and may provide a novel approach of using roflumilast in treating chronic autoimmune conditions such as RA.

P1224

Antiribosomal autoantibodies: three cases reported by a Madrilenian Hospital

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Purpose/Objective: In this work, we present the cases of antiribosomal autoantibodies observed in our department from March 2.010 to Abril 2.012. These autoantibodies are presented in 10−35% of patients with systemic erythematosus lupus (SLE) and they have been associated with a special form of SLE characterized by neurological and psiquiatric manifestations.
Materials and methods: From March 2010 to Abril 2012 were studied 8.005 consecutive sera samples received in our laboratory to be studied by indirect immunofluorescense (Mosaic: Basic Profile 3A, Euroimmune). Only 5 samples were considered as possible positive for antiribosomal autoantibodies and were confirmed by ImmunoLisa™ Ribosomal P Antibody Kit (Immco Diagnostics). PCR, Complement component C3 and C4 were quantified by nefelometry. Antinuclear specificities were studied by Luminex Technology.
Results: Three of the five sera that were suspected of anti-ribosomal autoantibodies were positive. Parallel analysis by Luminex Technology revealed their coexistence with other antinuclear antibodies such as, anti-SSB in case 1 and anti-RNP, -Sm, -dsDNA, -histones, in case 2. Anti- cardiolipin autoantibodies were positive in all the cases. The review of the clinical history of each patient revealed that these autoantibodies were present in patients with clinical manifestations of SLE, such as haematological manifestations (anemia: Hemoglobi n = 8.6 g/dl, trhombocytopenia: Platelets = 128.000/uL and leukopenia: Leukocytes = 2.200/uL) in the case 1 and a well defined nephritis lupic type V in case 2. The third case, a VIH-positive woman with no clinical manifestations of any autoimmune disease only elevated transaminases were reported (ALT = 61 U/l, AST = 62 U/l, GGT = 414 U/l). In none of them a neurological or psiquiatric manifestation was reported.
Conclusions: Antiribosomal autoantibodies are highly specific of patients with SLE. The patients studied in our laboratory present hamaetological or renal manifestations but not a neuropsiquiatric form typically associated with these autoantibodies. However, in the third case it is unknown that they play a special role or if are presented as a cause of a false cross-reaction against own antigens.

P1226

Association of MHC class III to animal models of rheumatoid arthritis

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Purpose/Objective: Rheumatoid arthritis (RA) is a multifactorial, chronic inflammatory joint disease. The HLA-DRB1 locus within the major histocompatibility complex (MHC) at 6p21.3 has been identified as a major susceptibility gene. Several human genetic association studies have suggested that, in addition to the DRB1, at least one other susceptibility gene for RA exist in the MHC class III. However, MHC class III has been difficult to study in human due to the strong linkage disequilibrium (LD) and high gene density. Our aim is, by isolating MHC class III congenic fragments in arthritis-prone DA/Han rats, to study the association of MHC class III to animal models of arthritis, independent of the HLA-DRB1 effect.
Materials and methods: Pristane-Induced Arthritis (PIA) and Oil-Induced Arthritis (OIA) were induced by pristane and incomplete Freund's adjuvant (IFA) respectively. Collagen-Induced Arthritis (CIA) was induced with type II collagen emulsified in IFA. TNF level was measured in peritoneal macrophages and blood by standard ELISA. In cell transfer experiment, IFA-primed inguinal lymph node cells were cultured and stimulated with concanavalin A for 65 h before transferring to F1 recipient rats.
Results: MHC class III congenic rat, DA.1HR56T, developed less severe disease ($P < 0.01$, day 13−20) with less weight loss ($P < 0.05$, day 16−20) compared to DA rats in OIA. Similar results were seen in PIA ($P < 0.05$, day 11−20) but not CIA. Despite the importance of TNF in the pathogenesis of RA and being one of polymorphic genes in the congenic fragment, we found no association between TNF and our disease phenotype. Adoptive transfer of T cells from OIA rats shows that this MHC class III locus controls the immune priming of the disease ($P < 0.01$, day 14−20). These disease phenotype differences are mapped to a 215-kb region in MHC class III. Real-time PCR identified MHC class III genes in the region with differential expression ($P < 0.01$) which could regulate the disease phenotype. We have recently split this 215-kb region further into 3 smaller subcongenic fragments which will take us closer to identifying the MHC class III arthritis-regulating gene.
Conclusions: We have shown the association of MHC class III to T-cell mediated arthritis disease models OIA and PIA, but not antigen-induced model CIA. We have presented the congenic approach to study arthritis in the gene-dense HLA class III which has traditionally been difficult to study in human.

P1227

Association of NLRP3 and CARD8 genetic polymorphisms with juvenile idiopathic arthritis in a Taiwanese population

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Purpose/Objective: Juvenile idiopathic arthritis (JIA) is a multi-genetic, chronic autoimmune disease of childhood. Elevated plasma IL-1βlevel had been observed in JIA, suggesting a possible role of inflammasome in pathogenesis. We determined if genetic polymorphisms of components of NLRP3 inflammasome confer risk for JIA.
Materials and methods: Of the 134 JIA patients and 103 healthy controls were genotyped for rs4353135 OR2B11/NLRP3 and rs2043211 CARD8 polymorphisms. Clinical laboratory data and serum IL-1β of JIA group were evaluated via medical chart review and ELISA, respectively.
Results: The variant rs4353135 G allele carrier had increased risk for JIA ($P = 0.036$) and elevated level of serum IL-1β ($P < 0.001$). Homozygous rs2043211 minor allele carrier was associated with the presence of rheumatoid factor (RF) ($P = 0.038$); while rs4353135 G/G genotype increased the need for Etarnercept (Enbrel) treatment ($P = 0.015$).
Conclusions: Our data suggest that functional genetic polymorphism affecting NLRP3 inflammasome components might contribute to the pathophysiology of JIA.

P1228

Cell-type specific type I interferon signatures in autologous stem cell transplanted lupus patients: IFN imprint remains in CD4+ T cells

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Purpose/Objective: Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease that affects multiple organs, whose pathology is mainly caused by the augmented interferon (IFN) signaling pathway. The aim of this study was to analyze the particular contribution of CD4+ T cells and monocytes with respect to cell type-specific IFN signatures detectable in SLE by global gene expression profiling. The major focus was set on the comparison of disease-active and -inactive patients either by standard drug treatment or by autologous stem cell transplantation (ASCT) that is assumed to completely reset the autoreactive immunologic memory.
Materials and methods: Affymetrix Human Genome U133 Plus 2.0 Array were made from purified peripheral CD4+ T cells from six active SLE, 2 inactive SLE by standard drug treatment and 3 inactive SLE who underwent ASCT as well as 3 healthy donors (ND). In addition, using the same donors, arrays were made from purified peripheral monocytes from one active SLE, 1 inactive SLE, 3 ASCT-treated SLE and 3 ND. A reference list of 2220 IFN pathway-related genes was obtained from a recent publication and used to estimate IFN imprints in SLE patients.
Results: In CD4+ T cells, it was obvious that inactive SLE showed a marginal IFN-imprint characterized by 233 only weakly expressed probe sets compared to active SLE characterized by 573 probe sets. Unexpectedly, 562 differentially expressed probe sets were also identified in ASCT-treated patients who are under long-term remission. However, considering the absolute magnitude of expression of IFN-regulated transcripts, the imprint in ASCT-treated patients was much weaker than in active SLE. It was obvious that monocytes showed a more complex IFN response characterized by 918 differentially expressed probe sets in active SLE. Different from CD4+ T cells, monocytes from ASCT-treated patients showed no apparent IFN signatures.
Conclusions: We could show for the first time detailed cell type-specific IFN signatures for CD4+ T cells and monocytes isolated from active and inactive SLE patients. Most interestingly, the intriguing question comes up, why only CD4+ T cells, but not monocytes of ASCT-treated patients, are characterized by an apparent IFN imprint although patients are under long-term remission. Our results indicate for a cell type-specific pro-inflammatory cytokine memory in T helper lymphocytes even after ASCT-therapy in SLE.

P1229

Comparison of synovial tissue cytokine mRNA profiles in very early arthritis patients with divergent outcomes

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Purpose/Objective: In the earliest clinically apparent phases of arthritis, it is difficult to distinguish patients who will develop rheumatoid arthritis (RA) from those whose disease will resolve. We sought to determine whether synovial tissue cytokine mRNA expression at presentation differed between early patients with these distinct outcomes.
Materials and methods: Synovial tissue biopsies were obtained within 12 weeks of symptom onset from 18 early synovitis patients who developed RA within 18 month follow-up, and 13 early synovitis patients with self-limiting disease. In addition, 12 patients with established RA, and 12 control subjects undergoing arthroscopy with no evidence of inflammatory pathology were studied. mRNA expression of 135 cytokines and related molecules was determined by real-time PCR using microfluidic cards. The Kruskal-Wallis test and principle component analysis were used for analysis. Synovial tissue sections were stained with haematoxylin and eosin and graded by infiltrate type. Synovial tissue sections were stained for CD3, CD4, CD8, CD68, CD20, CD15, mast cell tryptase and CD106 using immunofluorescence and cell counts were performed.
Results: Of the 135 cytokines analysed, 20 genes were differentially expressed between the 4 patient groups, 99 genes did not show significant differences, and 16 genes were not detected. Significant differences included higher levels of IFN-b and TNF in established RA compared to early RA, and higher levels of IL-2, IL-12b and IL-21 in established RA compared to early resolving synovitis. Principle component analysis showed early RA and resolving synovitis patients had overlapping cytokine mRNA profiles. mRNA expression of several cytokines correlated with leukocyte infiltrate grading and presence of leukocyte subsets. However, the type of leukocyte infiltrate and presence of leukocyte subsets were not significantly different between patient groups.
Conclusions: Cytokine mRNA expression patterns are similar in early synovitis patients who have resolving synovitis or develop RA. In addition, the clinical outcome of early synovitis patients is not reflected in the type of leukocyte infiltrate. These findings highlight the importance of alternative factors in determining resolution versus persistence in early synovitis.

P1230

Comparison of the peptide repertoires associated to hla-dr molecules differentially associated to rheumatoid arthritis

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Purpose/Objective: Rheumatoid arthritis (RA) is a chronic and systemic inflammatory autoimmune disorder that principally attacks synovial joints. The prevalence of RA is about 1% in the total population, being women more affected than men, in a ratio of approximately 2.3:1. The aetiology remains unknown, although it is known that environmental and genetic factors are involved in the pathogenesis of the disease. The strongest genetic association is with some human leukocyte antigens (HLA), including some subtypes of HLA-DR4 (DRB1*04:01, *04:04, *04:05 and *04:08), DR1 (DRB1*01:01) and DR10 (DRB1*10:01). Some alleles, as HLA-DR7 (DRB1*07:01) and DR15 (DRB1*15:01) are non-associated with the disease. A major feature shared by the RA-associated HLA*DR alleles is the presence of a basic consensus sequence in the third hypervariable region of DRB1, spanning residues 70–74 of the DRb chain. The presence of this basic 'motif' led to the proposal of the 'shared epitope' (SE) hypothesis, which postulates that the side chains of several of these amino acids could be involved in disease pathogenesis by either, defining the peptide preference or directly interacting with the T cell receptor (TCR), influencing the selection of the T cell repertoire. The objective of this work was to define if the peptide repertoires of HLA-DR associated molecules (DR1, DR4

and DR10) are more similar between them than with a non-associated molecule (DR15).
Materials and methods: HLA-DR molecules were immunoprecipitated from homozygous lymphoblastoid cell lines, using a mAb specific for a common epitope of the HLA-DR molecules. After acid elution, peptides were separated from HLA molecules by ultrafiltration. The peptide pool was fractionated by RP-HPLC in basic conditions and peptides were sequenced by mass spectrometry.
Results: A total of 843 peptides have been identified: 207 from DR1, 246 from DR4, 232 from DR10 and 158 from DR15. In these samples: DR10 shares 28 peptides with DR1, 10 with DR4 and 1 with DR15; DR15 shares 7 ligands with DR1 and 2 with DR4; finally, DR1 and DR4 bind 6 common peptides.
Conclusions: The direct mass spectrometry analysis of the HLA-DR natural ligands confirmed that the similarity between the peptide repertoires from DR1, DR4 and DR10 is higher that with that from DR15. Clearly, the two most similar peptide repertoires were those associated to DR1 and DR10, showing a prevalence of Leu en P4.

P1231
Cystamine ameliorates lupus-associated ventricular hypertrophy by modulating IL-6-mediated mitogen-activated protein kinase pathways

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Purpose/Objective: Left ventricular (LV) hypertrophy is prevalent in patients with systemic lupus erythematosus (SLE), possibly because of inflammation-related arterial stiffening. We previously demonstrated that cystamine ameliorates physiological functions of hepatocyte and macrophage in lupus-prone mice NZB/W-F1. Present study was aimed to investigate effect of cystamine on alleviation of LV hypertrophy in lupus mice and to elucidate underlying mechanisms.
Materials and methods: Hypertrophy was assessed by mass change. Tissue morphology was examined by histochemical staining and Masson's trichrome staining. Expression and phosphorylation of protein was determined and quantitated by immunoblotting using specific antibodies and densitometric analysis.
Results: Our results revealed that cystamine treatment decreased both LV mass and LV mass/tracheobronchial remnants (TBR) in NZB/W-F1 mice, whereas slight effects were observed in Balb/c mice. Moreover, cystamine treatment reduced levels of atrial natriuretic peptide (ANP), C-reactive protein (CRP), heart type-fatty acid-binding protein (h-FABP), creatine kinase-MB (CK-MB) and IL-6 in LV tissues of NZB/W-F1 mice. Additionally, in LV tissues of NZB/W-F1 mice, suppression of hypertrophic signaling mediated by IL-6 in response to administration of cystamine was revealed, including phosphorylation of MEK5, ERK5, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38).
Conclusions: Taken together, our results suggest that cystamine treatment alleviated LV hypertrophy in NZB/W-F1 mice as result of decrease in hypertrophic mediators and suppression of IL-6 mediated hypertrophic signaling. These findings indicate that cystamine has potential to be effective therapy for lupus associated cardiac hypertrophy in clinical setting.

P1232
Cytomegalovirus drives the expression of inhibitory NK cell receptor LIR1 on CD8+ T cells in RA patients

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Purpose/Objective: Rheumatoid arthritis (RA) is a chronic inflammatory disease which is accompanied by a dysregulated T cell homeostasis that leads to an expansion of autoreactive T cell clones. These cells show a late memory phenotype characterized by the loss of the costimulatory receptor CD28 and an increased expression of inhibitory natural killer (NK) cell receptors. LIR1 (also known as CD85j or ILT2) belongs to the family of inhibitory NK receptors; it is widely expressed among different immune cells and has been associated to autoimmunity before. Furthermore, it has been shown that a chronic viral infection like Cytomegalovirus (CMV), contributes to an expansion of autoreactive T cell clones. LIR1 seems to be increased in T cells in CMV+ as compared to CMV- healthy donors (HD).
Materials and methods: We were interested in the role of LIR1+ T cells in RA patients, which potentially contribute to the autoreactive T cell pool, especially in CMV+ patients. Therefore we investigated the frequency of LIR1 on T cells in peripheral blood mononuclear cells (PBMC) from patients with RA and HD. LIR1 and $CD28^-$ T cells on $CD8^+$ $CD3^+$ T cells were analyzed by flow cytometry.
Results: Flow cytometry analysis of 37 RA patients as well as 39 HD reveal a significant higher frequency of LIR1 on $CD8^+$ T cells ($P = 0.043$) in RA patients ($n = 37$, mean%: 9.43) compared to HD ($n = 39$, mean%:5.98).

Beyond that, we found a higher frequency of $CD8^+LIR1^+$ T cells in CMV+ compared to CMV- donors, in RA patients as well as in healthy controls. $LIR1^+$ T cell frequencies of CMV+ healthy controls ($n = 29$, mean%: 7.29) are significantly higher ($P = 0.017$) than in CMV- HD ($n = 10$, mean%:2.17). In RA patients this difference is even more pronounced ($P = 0.0027$), (CMV+ RA patients, $n = 32$, mean%: 10.6; CMV- RA patients, $n = 5$, mean%:1.74).

Importantly, we found a significant correlation ($P = 0.034$) of high numbers of $CD8^+LIR1^+$ with high disease activity score (DAS28) in RA patients without medical treatment ($n = 14$, $r = 0.568$). In addition also the numbers of $CD8^+LIR1^+$ T cells significantly ($P = 0.012$) correlate with the numbers of $CD8^+$ $CD28^-$ T cells in RA ($n = 37$, $r = 0.41$).
Conclusions: We are the first to demonstrate a significant increase of LIR1 expression on $CD8^+$ T cells of RA patients compared to HD. Furthermore this LIR1 expression profile associates with CMV infection in RA patients and is correlated to disease severity.

P1233
Cytotoxic T cell epitope in Brucellosis-induced murine spondyloarthropathy

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Purpose/Objective: Brucellosis is one of the most common bacterial zoonosis and causes significant human morbidity worldwide. Osteoarticular complications such as arthritis and spondylitis are the most frequent clinical manifestations. The goal of the study was to establish a murine model for human Brucellosis to better understanding the mechanisms of association between Brucellosis and spondyloarthropathy (SpA).
Materials and methods: BALB/c mice were infected with an intraperitoneal injection with 1−100 M CFU of the virulent bioluminescent *B. melitensis* strain GR023 or *B. abortus* vaccine strain S19. Mice were

sacrificed 3 and 26 weeks post-infection. Bacterial dissemination was monitored using biophotonic imaging and immunohistochemistry (IHC). Axial skeleton, paws, spleen, and liver tissue sections were stained for macrophage markers and for Brucella cell wall.
Results: *In vivo* biophotonic imaging showed accumulation of *B. melitensis* in paws and knees, but the strongest signal was detected in the axial skeleton. Upon IHC staining, intracellular bacteria were found in the spleen, liver, and bone marrow of peripheral joints and vertebrae. Three weeks post-infection, bacteria-infected cells were found in the subchondral bone marrow, but not present in the synovium, and infiltration by inflammatory cells was undetectable. Despite the general absence of infection in 26 weeks, mice developed arthritis in ankle and spine facet joints. We found that immunodominant Brucella-derived peptide from methionine sulfoxide reductase is presented by murine macrophages in context of MHC class I molecule to cytotoxic T cells. Homologous mouse/host protein carries a sequence that is 100% identical to Brucella epitope. Analysis indicates that Brucella epitope binds to human HLA-B27 class I molecules that are strongly associated with human SpA.
Conclusions: Arthritis was not associated with acute *Brucella* infection in this murine model. Inflammation in joints and changes in skeleton were found weeks after the bacteria were cleared from tissues. Murine Brucellosis demonstrated features that are typical to reactive arthritis with a potential involvement of cytotoxic T cells.

P1235
Effect of B CELL depletion by Rituximab in T cell subpopupulations from the peripheral blood of patients with rheumatoid arthritis

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Purpose/Objective: Rheumatoid arthritis (RA) is a chronic disease that leads to inflammation of the joints and other tissues. Rituximab (Rtx) is a therapeutic monoclonal antibody directed against CD20 that induces an important depletion of B cells and has been successfully used in RA. Although the efficacy of Rtx in RA is well documented, little is known about how it modifies the homeostasis of lymphoid subpopulations.

The objectives of the study were to know whether Rtxmodifies the ratio of the different T lymphocyte subpopulations from peripheral blood of RA patients.
Materials and methods: We studied 23 RA patients that underwent treatment with Rtx because of active RA and failure to at least one previous anti-rheumatic drug. The patients were divided into those naïve for Rtx treatment (Rtxn $n = 5$ patients) and those who had received at least one previous cycle of treatment with Rtx (Rtxs $n = 18$ patients). The treatment protocol consisted of two 1 g i.v. infusions, 2 weeks apart from each other. Peripheral blood samples for lymphoid subpopulation analysis were obtained at different times in both Rtxn and Rtxs patients: baseline (previous to the first infusion of Rtx) and at 3 and 6 months since baseline (t1 and t2 respectively) We studied different combinations of the following molecules: CD62L CD45RA CD45RO CD27 CD25 CD279 CCR5 CCR6 CCR7 CXCR5 CXCR6 that allowed us to calculate the percentage of memory and naïve T cells, effector and central memory T cells, follicular T helper cells and regulatory T cells, at each visit. Differences between groups were analyzed by ANOVA test.
Results: At baseline, there was a significant difference in the percentage of a considerable number of T cell subsets, standing out those regarding the memory and naïve differentiation stages. In particular, we observed a significant decrease of naïve T CD8 cells and a significant increase of memory T CD8 and memory T CD4 cells in Rtxs patients. During follow up period the differences in T cells observed at baseline became much smaller presumably because of the effects ofRtx, and no significant differences between the above mentioned T cell subsets were detected at t2.
Conclusions: Depletion of B cells through Rtx treatment leads to a profound change in the subpopulations of peripheral blood T lymphocytes, suggesting that mature B cells play a relevant role in the homeostasis of T cells in patients with RA.

P1237
Effect of Methotrexate in antigen-induced arthritis depends on regulatory T cells expression

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Purpose/Objective: Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the joints. The therapeutic approach of first choice for the RA treatment is the use of low doses of Methotrexate (MTX), an inhibitor of dihydrofolate reductase. Although its mechanism of action in RA is still poorly understood, MTX potentially acts via antiproliferative and anti-inflammatory means. Moreover, it is suggested that the suppressive effects of MTX in the disease may be related with suppressive activity of regulatory T (Treg) cells. Objective: To investigate the MTX effect on the population of Treg cells in experimental model of antigen-induced arthritis (AIA).
Materials and methods: mBSA-immunized C57BL/6 mice were pretreated with MTX (2 mg/kg) weekly for 5 weeks before challenge i.a. with 30 μg of mBSA at day 21 after first immunization. Articular hypernociception, cell migration, Treg cells frequency in the spleen and draining lymph nodes was evaluated 7 h after antigen challenge. Additionally, for depletion of Treg cells, mBSA-immunized C57BL/6 mice pretreated or not with MTX were undergoing administration of anti-CD25 (250 μg/ml) four times per week during the whole immunization protocol. After 7 h of the challenge with 30 μg i.a. of mBSA at 21 day, articular hypernociception was evaluated.
Results: MTX-treated animals showed an increase in hypernociception threshold and decrease in migration of neutrophils to the joint when compared to untreated animals. Interestingly, while the frequency of Treg cells ($CD4^+FoxP3^+$) in the draining lymph nodes were similar between groups; animals treated with MTX had an increase of this cell type in the spleen. In addition, Treg cells depletion with anti-CD25 monoclonal antibody treatment reversed the effect on articular hypernociception triggered by MTX.
Conclusions: These findings show that Treg cells are required for triggering anti-inflammatory effect of MTX in AIA model, and the drug still causes an increase of this cell population in treated animals.

P1238

Effect of therapy on the Th1/Th17 balance and cytokine profile in patients with juvenile idiopathic arthritis (JIA)

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Purpose/Objective: Juvenile idiopathic arthritis (JIA) is an inflammatory disorder with heterogeneous clinical presentation. Recently discovered IL-17-producing T-cells (Th17) have been identified as pro-inflammatory cells in several autoimmune conditions. This study evaluated the phenotype and the cytokine-production profile on Th1: IFNgamma and Th17:IL-17 cells in JIA patients with different treatments.
Materials and methods: T-cells of fifty-seven JIA patients (mean age: 12.9 ± 4.3 years) under therapy (non-steroidal-anti-inflammatory-drugs, ($n = 29$), disease-modifying-anti-rheumatic-drugs ($n = 25$), TNFalpha inhibitors ($n = 3$), and 14 JIA patients without therapy, and 28 healthy donors (HD) (mean age: 10.4 ± 4.4 years) were investigated by flow cytometry.
Results: IFNgamma-production by memory $CD4^+$ $CD45RO^+$ $CD28^+$ $CCR7^{+/-}$ (9.7%), $CD8^+$ $CD45RO^+$ $CD28^+$ $CCR7^{+/-}$ (8.5%), effector $CD4^+$ $CD45RO^+$ CD28-$CCR7^{+/-}$ (13.7%) and $CD8^+$ $CD45RO^+$ CD28-$CCR7^{+/-}$ (11.2%) T-cells were significantly lower in JIA patients than in HD (CD4+: 16.3%, 14.6%; CD8+: 27.2%, 18.2%, respectively) ($P < 0.05$). JIA patients showed a higher expression of IL-17 in memory (1.86%) and effector T-cells (1.02%) compared to HD (0.37%, 0.3%, respectively) ($P < 0.001$). IFNgamma-production was strongly reduced in effector T-cells in JIA patients under therapy (1.8%) compared to the non-treated JIA group (3.4%) ($P < 0.005$). IL-17-production in memory CD4+ T cells was lower in treated patients (0.44%) compared to non-treated patients (0.74%) ($P < 0.05$).
Conclusions: Overall, this study suggests an imbalance in the IFNgamma- and IL-17-production in memory and effector T-cell subsets in JIA patients. Whether specific therapy is able to permanently drive the cytokine profile to a less inflammatory function remains to be determined.

P1239

Effect of TLR8-deficiency on lupus pathogenesis in a spontaneous mouse model

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Purpose/Objective: SLE is a systemic autoimmune disorder characterized by increased B and Th cells activation, increased DCs presentation, by the formation of various autoantibodies and subsequent development of immune complex glomerulonephritis. The role of endosomal TLR7 and TLR9 in the development of anti-nuclear autoantibodies, because of their respective recognition of RNA and DNA, respectively, has been extensively studied. Little is known on the TLR8 activation *in vivo* in mice, we therefore conducted an innovating study on its possible implication in autoimmune responses in a murine model of lupus.
Materials and methods: To better define the contributions of TLR8 to the development of SLE, we introduced the TLR8 null mutation into C57BL/6 mice congenic for *Nba2 (NZB autoimmunity 2)* locus (B6.Nba2) and followed the development of the disease, B, T cells and DCs activations, autoantibody production and mortality due to glomerulonephritis.
Results: TLR8-deficiency resulted in an accelerated SLE characterized by increased total IgM and IgG levels in the sera, increased MHCII expression on APCs and higher tlr7 mRNA expression on APCs. DC capacity to present allogenic antigens to CD4 T cells was also improved after TLR7 ligation.
Conclusions: TLR8 deficiency in lupus-prone mice induces acceleration of SLE due to up-regulation of TLR7-dependent APC activation. DCs function seems to be the key player of the TLR8 deficiency effect.

P1240

Elevated soluble E-cadherin levels in chronically inflamed joints favour TNF production by KLRG1 expressing T cells

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Purpose/Objective: The killer cell lectin-like receptor G1 (KLRG1) is a NK cell marker that is also expressed on antigen-experienced T cells showing an immune senescent phenotype. KLRG1 binding to its ligand E-cadherin results in inhibition of cytokine-producing and cytotoxic T cell responses. Recently, the soluble form of E-cadherin (sE-cadherin) has been shown to influence KLRG1 signaling. Furthermore, it has been hypothesized that senescent T cells play a role in development of autoimmunity but the potential involvement of KLRG1 in an arthritic/rheumatic disease has not been investigated yet.
Materials and methods: PBMC/SFMC from 21 chronic arthritis patients [rheumatoid arthritis (RA) or spondyloarthritides (SpA)], 8 patients with crystal induced acute arthritis (gout and chondrocalcinosis) and 10 healthy controls were obtained. T cells were characterized for KLRG1 expression directly *ex vivo*, while TNF/IFN-γ-production was assessed after 4 h PMA/CaI stimulation by flow cytometry. In addition, sE-cadherin levels in paired plasma − SF were determined. Moreover, TNF/IFN-γ production by T cells was compared in the presence/absence of sE-cadherin in a 7-day *in vitro* culture system.
Results: More T cells were KLRG1+ in the SF as opposed to the PB of patients with chronic arthritis (RA and SpA), which contrasts strikingly with results obtained in crystal induced arthritides. The KLRG1+ T cell subset had a functionally more active phenotype, characterized by increased capacity to produce proinflammatory cytokines such as TNF or IFN-γ. Levels of sE-cadherin were found to be markedly higher in the SF of all arthritides. Unexpectedly, the presence of sE-cadherin enhanced TNF but not IFN-γ production by KLRG1+ T cells.
Conclusions: Both KLRG1+ T cells and its ligand sE-cadherin were increased in the SF of chronic arthritis patients. Surprisingly, sE-cadherin is likely to contribute to the local proinflammatory environment in the joint by favouring TNF production by KLRG1+ T cells. Importantly, this pathway seems to be operational in both RA and SpA, but not in acute crystal induced forms of arthritis.

P1241

Enhanced megakaryopoiesis as a pro-pathogenic factor in a mice model for Systemic Lupus Erythematosus

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Purpose/Objective: Autoantibodies contribute to the pathogenesis of the autoimmune disease Systemic Lupus Erythematosus (SLE) by stimulating the immune response. Further, deposits of immune complexes in the kidneys can lead to severe nephritis. The source for autoantibodies is short- and long-lived plasma cells located in the bone marrow and -differently to protective plasma cells − in the spleen. In

NZB/W mice – a mouse model for SLE – both parental strains New Zealand Black (NZB) and New Zealand White (NZW) contribute different *sle-loci* to the formation of SLE. The NZB strain passes the *sle2c locus* in which the gene for the Thrombopoietin (TPO)-receptor (*c-mpl*) is located. According to the relevance of megakaryocytes for the plasma cell niche and the correlation between plasma cell and megakaryocyte numbers as reported earlier by us, we wanted to elucidate whether c-Mpl and/or megakaryopoiesis is altered in auto-immune mice.

Materials and methods: Therefore, we examined in wild type, NZB and NZW mice the amount of megakaryocytes in spleen and bone marrow, the occurence of genetic variations for *c-mpl* and the degree of megakaryopoiesis upon TPO stimulation by histological survey, flow-cytometric and genetic analysis and by *in vitro* studies.

Results: We found, that in the spleen of NZB mice plasma cell and megakaryocyte numbers are 10-times higher than in wild type while in NZW mice the numbers are equal. We detected a missense mutation in the *c-mpl* gene of NZB mice leading to an amino acid replacement within the essential TPO-binding site. Upon TPO stimulation of spleenocyte and bone marrow cultures NZB cultures responded significantly stronger resulting in the double amount of megakaryocytes compared to NZW cultures.

Conclusions: In summary, our data indicate that the mutated *c-mpl* gene located within the *sle2c-locus* is leading to an augmented megakaryopoiesis which enables the accumulation of a greater number of autoreactive plasma cells and thus is contributing to the development of SLE.

P1242
Evidence for spontaneous type I interferon release in SAMHD1 deficient mice

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Purpose/Objective: The term 'interferonopathies' designates a group of diseases in which an uncontrolled chronic type I interferon (IFN) response is considered a key pathogenic mechanism, a prominent member of this group being systemic lupus erythematosus (SLE). Also Aicardi-Goutières syndrome (AGS), an early-onset inflammatory encephalopathy which clinically and pathogenetically overlaps with SLE, is characterized by IFN production in the absence of detectable viral infection. AGS is caused by genetic defects of any of three intracellular enzymes, Trex1, RNase H2 or SAMHD1, all involved in intracellular nucleic acid metabolism. The finding that Trex1-deficient mice develop typ I IFN-dependent autoimmune multi-organ inflammation stimulated a new concept of autoimmunity caused by intracellular nucleic acid accumulation, which triggers IFN production. SAMHD1, a phosphohydrolase which cleaves dNTPs and thereby prevents retroviral (HIV) reverse transcription in human cells. Our aim was to elucidate the pathogenesis of SAMHD1-associated AGS.

Materials and methods: We generated mice with complete deficiency for SAMHD1.

Results: SAMHD1$^{-/-}$ mice were macroscopically normal and aged without any sign of systemic autoimmunity. We did not find evidence for SAMHD1-dependent retroviral restriction *in vitro* or *in vivo*. However, SAMHD1$^{-/-}$ peritoneal macrophages showed a type I IFN-induced gene expression signature indicative of spontaneous type I IFN production, similar to the interferon signature of Trex1$^{-/-}$ mice.

Conclusions: Although SAMHD1 deficiency in mice is not sufficient to trigger systemic autoimmunity, the animals recapitulate an early key feature of AGS pathogenesis, which is likely relevant also for SLE. We think that the animals will be useful for the investigation of mechanisms precipitating a pathogenic IFN response.

P1243
FKBP, a new gene involved in systemic lupus erythematosus?

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Purpose/Objective: Systemic lupus erythematosus (SLE) is a severe autoimmune disease, characterized by the production of various autoantibodies and multiorgan damages. SLE involves both genetic and environmental factors. Several studies have mapped some autoimmune susceptibility regions in the genome in humans and mice, but most of the candidate genes are disputed or unconfirmed at this time. Several arguments strongly suggest that genetic defects in B cells pre-exist in the development of the disease. However, only a few genes among the published SLE susceptibility genes are linked to B cell functions. We propose to characterize one genetic intrinsic B cell abnormality found in a group of lupus patients, by functional genomics studies.

Materials and methods: We have performed a B cell transcriptoma analysis in quiescent SLE patients in comparison to normal subjects. A short list of interesting over- or under-expressed genes has been established. We pointed an overexpression of a member of FKBP peptidyl/prolyl cis/trans isomerases family (here named 'FKBPO'), which catalyzes the folding of proline-containing polypeptides. This family can regulate cellular mechanisms due to conformational changes of key proteins. The role of this protein in B cells is completely unknown. Thus we developed lentigenic mice overexpressing FKBP in order to reproduce the variation of gene expression that we observed in patients, and to evaluate its effect on B cell phenotype and lupus development.

Results: The analysis of B cell phenotype in 8 months-old FKBP lentigenic mice has shown: (1) an increase of total cell number in spleen and lymph nodes, (2) a higher production of IgG3, (3) a production of autoantibodies in subsets of animals. Moreover we observed a higher B cell response *in vivo*, by immunization with a T-cell independent antigen. Finally, the differentiation of B cells into plasmocytes is increased in FKBP lentigenic mice, compared to control mice, after stimulation with LPS *in vitro*.

Conclusions: Our results show that the overexpression of FKBP increases the risk to develop autoimmunity. Experiments are in progress in order to evaluate if FKBP overexpression can exacerbate the development of autoimmunity in autoimmune-prone mice or can modify the establishment of B cell tolerance in a transgenic model of B cell tolerance.

P1244
Foxp3+ regulatory T cells that lack CD25 expression might serve as a marker to diagnose and monitor Systemic Lupus Erythematosus (SLE) patients with kidney involvement

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Purpose/Objective: $CD4^+$ $CD25^-$ $Foxp3^+$T cells have been shown to be a population of regulatory T cells which are increased in SLE pa-

tients. So far their detailed role in SLE patients has not been elucidated. We therefore investigated the role of disease activity, treatment and organ involvement on proportions of $CD4^+$ $CD25^-$ $Foxp3^+$T cells in SLE patients.

Materials and methods: Percentages of $CD4^+$ $CD25^-$ $Foxp3^+$T cells-were determined in peripheral blood mononuclear cells (PBMC) in HC ($n = 21$) and SLE patients ($n = 61$) by FACS. In selected SLE patients suffering from active glomerulonephritis, proportions of $CD4^+$ $CD25^-$ $Foxp3^+$T cells were analyzed in urine samples. Percentages of $CD4^+$ $CD25^-$ $Foxp3^+$T cells were correlated with clinical data, therapy and disease activity indices. Time course analyses of proportions of $CD4^+$ $CD25^-$ $Foxp3^+$T cells were performed in patients suffering from active glomerulonephritis before and after treatment with cyclophosphamide and in patients with active skin involvement before and after cortisone treatment.

Results: Proportions of $CD4^+$ $CD25^-$ $Foxp3^+$T cells were significantly increased in patients as compared to HC and proportions of $CD4^+$ $CD25^-$ $Foxp3^+$T cells were significantly higher in active as compared to inactive patients. We observed a significant correlation of % $CD4^+$ $CD25^-$ $Foxp3^+$ T cells with the disease activity scores SLEDAI, ECLAM and SIS and with the daily cortisone dose. Time course analysis revealed no influence of cortisone treatment on the percentage of $CD4^+$ $CD25^-$ $Foxp3^+$T cells in patients with active skin involvement, whereas cyclophosphamide treatment of patients with active glomerulonephritis led to a decrease in $CD4^+$ $CD25^-$ $Foxp3^+$T cells. Further analyses of the different organ involvements revealed that only patients with renal involvement had significantly higher proportions of $CD4^+$ $CD25^-$ $Foxp3^+$T cells among SLE patients. Proportions were even increased in active nephritis patients as compared to patients with chronic kidney disease. Furthermore we observed a significant correlation between proportions of $CD4^+$ $CD25^-$ $Foxp3^+$T cells and the extent of proteinuria and could detect $CD4^+$ $CD25^-$ $Foxp3^+$T cells in urinary samples of patients suffering from active nephritis.

Conclusions: Proportions of $CD4^+$ $CD25^-$ $Foxp3^+$T cells are significantly increased in SLE patients with renal involvement. Since $CD4^+$ $CD25^-$ $Foxp3^+$T cells also correlate with the extent of proteinuria and were found to be decreased after cyclophosphamide treatment in long time course analyses, $CD4^+$ $CD25^-$ $Foxp3^+$ T cells might represent a new biomarker to recognize and monitor patients with renal involvement.

P1245
Functional analysis of a genetic variant of ITGAM (CD11b/Mac-1) associated with systemic lupus erythematosus

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Purpose/Objective: Systemic lupus erythematosus (SLE) is a clinically diverse autoimmune inflammatory disease, with a strong genetic component. There is no cure for SLE and treatment often utilises antimalarial and anti-inflammatory drugs, both of which can cause deleterious health effects with prolonged use. Therefore, greater understanding of SLE pathogenesis in individual cases may lead to the development of more specific therapies that focus on the underlying causes of the disease and reduce reliance on anti-inflammatories.

Our research concerns a genetic variant of the ITGAM (CD11b) gene that has been found to be associated with the incidence of SLE. The ITGAM gene codes for CD11b which is the alpha-chain of the Mac-1 (CD11b/CD18) integrin. This integrin is expressed on myeloid cells and binds several ligands, including members of the ICAM family and the complement factor iC3b. It is involved in immunological processes, such as leukocyte extravasation and phagocytosis. In addition, Mac-1 has been described to negatively regulate immune cell signalling. The SLE-associated genetic variant in ITGAM leads to a single amino acid substitution at position 77 in the extracellular domain of the integrin where an arganine is replaced with a histidine (R77H CD11b).

Materials and methods: We have used R77H CD11b transfected cells and primary human monocytes isolated from buffy coats to analyse CD11b surface expression, activation state, adhesion to ligands, phagocytosis and cytokine production.

Results: We have shown using R77H-CD11b transfected cells that this amino acid substitution does not affect the surface expression of the integrin. However the function of the integrin is severely impaired. R77H CD11b expressing cells display defects in cell adhesion to ICAM-1 and iC3b and phagocytosis of iC3b coated beads. In addition, R77H-CD11b transfected cells display increased IL-6 production as compared to WT-CD11b expressing cells.

Conclusions: We are currently isolating leukocytes from buffy coats from a healthy population, and examining Mac-1-dependent functions in these cells to esablish if the H77 alelle is associated with defects in hese cells. The H77 allele occurs at a frequency of 1 in 10 in the European population so we expect to be able to obtain sufficient heterozygotes and homozygote H77 monocytes to compare with the R77 variant (R77). It is hoped that this work will ultimately provide an insight into the pathogenesis of SLE.

P1246
G-modified inhibitory oligonucleotides for treatment of TLR-mediated autoimmune diseases such as systemic lupus erythematosus

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Purpose/Objective: Inappropriate or excessive activation of TLR7/8 and 9 by self RNA or self DNA complexes is presumably involved in the pathogenesis of SLE. Stimulation of both TLRs activates pDCs to secrete IFN-alpha and autoreactive B-cells to produce autoantibodies. Up to date, there are rather unspecific immune modulators available for treatment, such as corticosteroids and cytostatics. These drugs are limited in their efficiency and side effects restrict their use. Inhibitory synthetic oligonucleotides (INH-ODN) block DNA-mediated TLR9 and RNA-mediated TLR7/8 stimulation, and can prevent autoimmunity as shown in several lupus mouse models. However, many INH-ODNs generate G4-stacks which lead to higher order structures making them unsuitable for further pharmaceutical development. Our goal was to design INH-ODNs which do not form these structures without losing any inhibitory capacity.

Materials and methods: We modified guanosine residues of INH-ODNs in such a way that G4-stacks were no longer formed and compared their efficiency to classical INH-ODNs *in vitro* and *in vivo*.

Results: Upon stimulation of TLR7 and 9, these new INH-ODNs inhibited the secretion of TNF-alpha and IL-12 by bone marrow derived-macrophages (BMDM) and -dendritic cells (BMDC) *in vitro* and were at least as effective as the classical INH-ODNs. Furthermore, they appeared to be more potent in the inhibition of cells considered as relevant in autoimmunity. Thus, INF-alpha secretion by plasmacytoid dendritic cells and IL-6 secretion and proliferation of B-cells derived from WT and lupus-prone MRL^{lpr} mice was more efficiently inhibited compared to classical INH-ODNs. Our most promising candidate based on our *in vitro* studies, INH-ODN 24888, was also able to inhibit TLR7- and TLR9-mediated stimulation *in vivo* in Balb/c mice more potently than the established INH-ODN 2088. Additionally, we could show that mucosal application of INH-ODNs was sufficient to prevent systemic consequences of CpG stimulation and INH-ODN 24888 appeared again to be more effective than INH-ODN 2088.

Conclusions: In conclusion, our new modified INH-ODNs do not form unfavorable higher orders and show higher inhibitory capacity *in vitro* and *in vivo*.

P1247
GARP and miR142-3p expression in patients with rheumatoid arthritis

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Purpose/Objective: The function of regulatory T cells (Tregs) is diminished in patients with autoimmune diseases, such as rheumatoid arthritis (RA). GARP is a surface protein specifically expressed on activated Tregs. Diminished expression of GARP attenuates the function of Tregs. In a previous study, we could document that GARP expression is regulated by miR142-3p. To evaluate whether reduction of Treg function in RA might be caused by insufficient regulation of GARP expression by miR142-3p, we examined the expression of miR142-3p and GARP in patients with RA.
Materials and methods: CD25+ CD4 T cells were isolated from the peripheral blood of early, treatment naïve RA patients ($n = 6$) and healthy individuals (HC) ($n = 16$). The cells were stimulated for 2 days with anti-CD3/CD28 Dynabeads. GARP surface expression was assessed by FACS staining on days 0, 1 and 2. Total RNA was isolated daily. GARP mRNA and miR142-3p expression were assessed using TaqMan gene expression and miRNA expression assays.
Results: Consistent with the previous findings, CD25+ Tregs purified from RA patients and from healthy individuals did not express detectable GARP levels on the surface. In response to activation, both Treg populations upregulated GARP. The upregulation was, however, greater in CD25+ Tregs from RA patients. At day 0, expression of miR142-3p was comparable between Tregs from HCs and RA patients. Upregulation of miR142-3p in response to stimulation was, however, again greater in cells from the patients.
Conclusions: The data indicate a deregulated expression of GARP and miR142-3p in RA and suggest that so imbalanced miR142-3p expression might influence GARP expression on Tregs and thereby the function of Tregs in RA.

P1248
Genetic associations to germinal centre formation in primary Sjögren's syndrome

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Purpose/Objective: Primary Sjögren's syndrome (pSS) is an autoimmune rheumatic disease mainly affecting the salivary and lacrimal glands causing xerostomia and keratoconjunctivitis sicca. Focal mononuclear cell inflammation in the form of germinal center-like structures (GC) is found in the minor salivary glands of 20–25% of patients. We have previously shown that GC+ pSS patients presented with elevated serum levels of IL-1RA, IL-4, IL-17 and MCP compared to GC$^-$patients. In this follow-up study, we aimed to assess the genetic variations in GC+ and GC$^-$ pSS patients.
Materials and methods: In a Swedish-Norwegian pSS cohort ($n = 540$), GC+ ($n = 76$) and GC$^-$ ($n = 244$) patients were identified. Of the 1536 single-nucleotide polymorphisms (SNPs) were analysed in whole blood DNA by the Illumina GoldenGate assay (Illumina Inc.). Minor allele frequencies in GC+ and GC$^-$ patients were compared using Fisher's exact test and associations were considered significant when $P < 0.001$ and suggestive when $P < 0.01$. Statistical analysis were performed using the PLINK software.
Results: In this case-only analysis of 320 pSS patients with known GC status, we identified two SNPs in *Eotaxin* associated with GC+ patients with OR 0.45 and 0.41. Furthermore, we found suggestive associations with *BANK-1*, *ICA-1*, *IL-17, PRKCL1, CARD-8, Bcl-2, TANK, IKBKE, AID* and *APRIL*. We also detected weak associations ($P < 0.05$) with SNPs in the BLK, STAT1, STAT4, SSA1, SSB, IL15RA, IL-6 and TNF-a genes. Serum eotaxin (CCL11) has previously been identified as a key discriminator between GC+ and GC$^-$ pSS patients. The formation of GCs depends on B cell stimulation by helper T cells via the CD40-CD40L system, which also contribute to the expression of activation induced deaminase (AID). Genetic variations in *CD40L* and *AID* suggest that GC+ patients may be genetically predisposed for ectopic GC formation. B lymphoid kinase (BLK) has a role in the development and activation of marginal zone B cells present in the GC formations, and Bcl-2 is an anti-apoptotic protein which gene is implicated in a number of cancers and autoimmunity. CARD-8, IKBKE and TANK are regulators of the NF-κB pathway, a pathway with a well-established role in secondary lymphoid organ development.
Conclusions: Taken together, our findings suggest that genetic variations may help explain why ectopic GC-like structures are present in some pSS patients but not all.

P1250
Hypoxia and hypoxia-inducible factor-1a exacerbate Toll-like receptor signalling-engaged inflammatory response in rheumatoid arthritis synovial fibroblasts

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Purpose/Objective: Hyperplasia of synovial fibroblasts, infiltration with lymphocytes, and tissue hypoxia are the major characteristics of rheumatoid arthritis (RA). Much data support a key role for toll-like receptors (TLRs) in RA. Little is known regarding the impact of hypoxia on TLR signalling-induced inflammatory response in RA. The aim of this study was to reveal the effect of hypoxia and its regulator hypoxia-inducible factor-1α (HIF-1α) on the inflammatory response in rheumatoid arthritis synovial fibroblast (RASF) upon the recognition of pathogen molecules.
Materials and methods: Hypoxia was induced in RASF by incubation with $Na_2S_2O_4$. The TLR3 ligand polyIC, TLR2 ligand PGN, TLR4 ligand LPS, and TLR9 ligand CpG were used to stimulate the cells. Effects of hypoxia on TLR-induced inflammatory cytokines and matrix metalloproteinases (MMPs) were determined by RT-PCR, real time PCR, and ELISA. Overexpression of HIF-1α as well as knocking-down its expression by siRNA were used to reveal its fundamental role. RASF-induced inflammatory T cell population expansion was determined by flow cytometry, real time PCR, and ELISA analyses after RASF/T cell co-culture.
Results: Hypoxia potentiated the expression of inflammatory cytokines, MMPs, and VEGF in RASF stimulated by different TLR ligands, especially polyIC, a synthetic mimic of dsRNA from virus or apoptotic cells. HIF-1α played a fundamental role in this synergy. Moreover, HIF-1α overexpression enhanced RASF-mediated inflammatory T cell proliferation, inducing more proinflammatory IFN-γ and IL-17 production.
Conclusions: Our findings suggest that hypoxia and HIF-1α may function in conjunction with TLR-engaged innate immune responses to exacerbate the pathogenesis of RA. This pathway may serve as a therapeutic target for disease.

P1251

IFNa enhances TNFa-stimulated production and STAT4 expression in monocytes. *In vivo* and *in vitro* effect of antimalarial drugs

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Purpose/Objective: IFNα plays an important role in the etiopathogenesis of systemic lupus erythematosus (SLE) and other autoimmune diseases and is involved in the production of several pro-inflammatory cytokines in humans. Thus, we wanted to analyse the influence of IFNα on the production of TNFα by human peripheral blood mononuclear cells (PBMCs) as well as the possible interference of this cytokine on the effect of antimalarial drugs, TNFα inhibitors widely used in the treatment of SLE.
Materials and methods: PBMCs were incubated with IFNα (1000 U/ml), alone or in combination with other stimuli (PHA, LPS), as well as in the presence or absence of chloroquine. Supernatants from such cultures were collected to quantify TNFα levels by ELISA, while TNFα and STAT4 expression in cultured cells were analysed by intracellular flow cytometry. In addition, TNFα and IFNa serum levels were quantified in 171 SLE patients and 109 healthy donors by ELISA and STAT4 expression was analyzed in 60 SLE patients and 25 controls by real time RT-PCR.
Results: IFNα treatment of PBMC did not induce a significant increase of the TNFα secretion respect to untreated cells; however, this cytokine produced a strong increase in TNFα levels in LPS or PHA-stimulated cells. Moreover, when we investigated the cellular population responsible of this effect we detected an increased secretion of TNFα by stimulated monocytes but not by T lymphocytes. In addition, we observed an increase of the intracellular STAT4 levels in monocytes stimulated with LPS in the presence of IFNα, suggesting that this effect could be mediated by STAT4. The analysis of antimalarial effect showed that chloroquine was able to inhibit the expression of TNFα enhanced by IFNα in activated cells as well as the STAT4 levels. Finally, the analysis of SLE patients, which presented a positive correlation between IFNα and TNFα serum levels, showed that antimalarial treatment, in addition to decrease TNFα, could be able to reduce STAT4 gene expression but only in patients with high IFNα serum levels.
Conclusions: IFNα treatment in *in vitro* cultures enhances the induction of TNFα by stimulated monocytes, an effect that seems to be mediated by STAT4 and inhibited by chloroquine treatment. However, the consequence of antimalarial treatment on SLE patients could be different depending on their IFNα serum levels.

P1252

IFNa serum levels are associated with poor prognosis markers, Endothelial Progenitor Cells imbalance and proinflammatory cytokine profile in Rheumatoid Arthritis patients

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Purpose/Objective: As a role for IFNα in endothelial damage and endothelial progenitor cell (EPC) disturbances in autoimmune disorders has been proposed, the main aim of this study was to evaluate the potential associations of IFNa serum levels on EPC populations and cytokine profiles in RA patients.
Materials and methods: EPC populations were quantified by flow cytometry in peripheral blood samples from 50 healthy controls (HC) and 100 RA patients based on their CD34, VEGFR2 and CD133 expression. Cytokine serum levels were measured by immunoassays and clinical and immunological data, including cardiovascular events and cardiovascular risk factors, were obtained by reviewing clinical records.
Results: We found that IFNα serum levels were associated with EPC populations in RA. However, only 33% of patients displayed high levels of this cytokine, using the percentile 90 in HC as cut-off. In fact, patients with low IFNa levels displayed a significant EPC depletion, whereas the IFNα-high group exhibited higher mature EPC (mEPC) and EC counts, and an increased mEPC/EPC ratio. Moreover, high IFNα serum levels were associated with higher disease activity (DAS28), poor prognosis markers (Rheumatoid Factor and anti-CCP autoantibodies) and higher levels of proinflamatory cytokines (IL-1β, IL-12 and IL-6) accompanied with lower amounts of VEGF, thus suggesting higher rates of endothelial damage and an endothelial repair failure. Finally, the increased frequency of cardiovascular events found in patients with high IFNα levels ($P < 0.05$) seems to support this hypothesis.
Conclusions: IFNα serum levels could isolate a group of RA patients with an enhanced proinflamatory profile which may impair endothelial repair through an EPC imbalance, thus leading to higher cardiovascular risk.

P1254

IL-6 trans-signaling drives collagen production in fibroblasts

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Purpose/Objective: In this study, we set out to determine the role of Signal Transducer and Activator of Transcription 3 (STAT-3) in the pathogenesis of systemic sclerosis (SSc), a rare connective tissue disease characterized by vasculopathy, inflammation, and fibrosis.
Materials and methods: Healthy dermal fibroblasts were cultured from a healthy donor after punch biopsy of involved skin. Fibroblasts were treated with IL-6 (25 ng/ml) andsIL-6R (20 ng/ml) alone and a combination of both cytokines. Collagen I transcripts were quantified by Q-RT-PCR. We also performed immunocytochemistry, using antibody against α-SMA, and Western blot analyses to determine whether STAT-3 and Smad3 phosphorylation were affected by *in vitro* culture of fibroblasts with IL-6 and/or sIL-6R.
Results: These experiments showed that the combination of IL-6 and sIL-6R induced the strongest collagen fold increase, which was 7.76 times higher ($n = 2$) compared to control (untreated fibroblast). Western blot analysis confirmed the direct contribution of IL-6 in STAT-3 phosphorylation and indirect contribution in Smad3 phosphorylation. Immunocytochemistry showed a direct effect of IL-6 on α-SMA levels.
Conclusions: We conclude that IL-6 trans-signaling affects collagen expression in dermal fibroblasts and postulate that a similar mechanism is operative in SSc.

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Incidence and significance of Antibodies to ribosomal P proteins in lupus without neuropsychiatric manifestations

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Purpose/Objective: Several studies showed a strong association between elevated titers of serum anti-ribosomal P antibodies andneuropsychiatric manifestations (NM) of Systemic Lupus Erythematosus (SLE). The aim of this work is to assess frequency of anti-ribosomal P

proteins antibodies and then study their clinical significance in SLE without NM.

Materials and methods: Eighty SLE patients without NM were included in this study. Among them 47SLE patients with biopsy-proven nephritis. Clinical activity was measured according to the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI index). For detecting antibodies to ribosomal P proteins, serum samples were tested using the multiplex method (Luminex and AtheNA Multi-Lyte).

Results: Sera from 24 patients were positive for anti-P, which represents 30% of our patients. This rate is consistent with the literature data. Among these 24 positive subjects, 17 of them have renal impairment (17/24) whereas only 6 of patients (6/33) without renal involvement were positive (36%vs18%, $P < 0.01$). Stratification of positive anti-P patients with lupus nephritis according to disease activity revealed that 14 from the 17 one of these categories of patients are in clinical relapse (74%vs26%, pc < 0.01). Our findings correlated with the results of some studies showing that these antibodies represent a marker of lupus nephritis flare.

Conclusions: Even if antibodies to ribosomal P proteins occur in approximately one third of lupus patients without MN, they seem to have high specificity for monitoring lupus nephritis flare. However, their low sensitivity is the main parameter limiting their search in daily practice.

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Increased genomic ribonucleotide content and spontaneous DNA damage response in mice with defects of the Aicardi-Goutières syndrome-associated endonuclease RNase H2

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Purpose/Objective: RNases H cleave the RNA moiety of RNA/DNA hybrids. RNase H2 mutations cause Aicardi-Goutières Syndrome (AGS), a neonatal inflammatory disease, which shows clinical overlap with systemic lupus erythematosus (SLE). Like SLE, AGS patients are characterized by a spontaneous type I IFN response, which presumably plays a key role in pathogenesis.

Materials and methods: We generated mice with complete or cell type-specific RNase H2 deficiency or with hypomorphic expression of the enzyme.

Results: We show that RNase H2 deficiency is embryonic lethal. RNase H2-deficient cells show proliferative impairment and accumulate in G2/M phase of the cell cycle due to a spontaneous DNA damage response. Embryonic fibroblasts with reduced RNase H2 activity featured increased frequency of genomic single strand breaks, increased numbers of nuclear gH2AX foci and induction of p53-target genes, most prominently the cyclin-dependent kinase inhibitor 1 gene encoding cell cycle inhibitor p21. DNA from RNase H2-deficient embryos featured an increased load of ribonucleotides in the genomic DNA. These findings suggest that unrepaired ribonucleotides trigger the DNA damage response in RNase H2-deficient cells.

Conclusions: Collectively, we show that RNase H2 is essential to remove ribonucleotides from the mammalian genome to prevent DNA damage. Our findings raise the question, whether a chronic DNA damage response is of relevance in the pathogenesis of AGS and related conditions including SLE.

P1257

Inflammation and bone erosion in the delayed-type hypersensitivity (DTH) arthritis model

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Purpose/Objective: Rheumatoid arthritis (RA) is a chronic inflammatory and destructive autoimmune disease, characterised by joint inflammation and bone erosion. To better understand the underlying immune mechanisms of RA various models of arthritis have been developed in different inbred mouse strains. Delayed-type hypersensitivity (DTH) arthritis is a mono-paw arthritis model in C57BL/6 mice affecting one hind paw. DTH-arthritis is induced by modifying a protein antigen induced DTH response by administration of anti-type II collagen antibodies between immunisation and challenge. Onset is synchronised, incidence is ≥100% and intra-group variation is low. The mono-paw arthritic phenotype makes the model suitable for analysis of inflammation and bone destruction, as the unaffected control hind paw can be used as an intra-animal control. The purpose of this study was to improve our understanding of DTH arthritis-associated inflammation and bone destruction by exploring different biomarkers of disease using various state of the art techniques.

Materials and methods: Leukocyte subsets were analysed by flow cytometry, and paw homogenates and serum by ELISA/Bioplex. A gene expression analysis was performed using deep sequencing (mRNA expression) techniques. Fluorescence molecular tomography was used to *in vivo* image enzyme activities and bone remodeling. Finally, a histopathological scoring system was established to assess pathological changes in the affected paw.

Results: In the affected paw, we observed marked infiltration of neutrophils and macrophages as well as osteoclast activation, bone erosion and remodelling, cartilage destruction and increased activity of enzymes associated with these processes. Serum analysis revealed an increase of systemic levels of several inflammatory markers. Analysis of paw homogenates revealed an increase of several markers associated with inflammation and bone erosion. Moreover, gene expression analysis revealed an increase in mRNA expression of several genes associated with inflammation and bone erosion.

Conclusions: We have performed analyses of biomarkers for disease in DTH arthritis, which indicate that DTH arthritis shares features with RA. We believe that the applied state of the art techniques provide highly valuable model insights from which efforts into development of novel biologics targeting RA can benefit.

P1258

Intrarenal FOXP3+ regulatory T cells expansion and decreased number of infiltrating CD4+ T cell in murine lupus nephritis by IL-2 therapy

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Purpose/Objective: Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by an acquired IL-2 deficiency, which leads to a homeostatic imbalance between regulatory T cells (Treg) and effector T cells (Tcon). Humrich *et al.* (2010) demonstrated that the IL-2 deficiency in diseased NZB/W mice can be rebalanced in lymphoid organs using a treatment with recombinant IL-2 (IL-2) by promoting the homeostatic proliferation of regulatory T cells. The aim of this study was to investigate the impact of IL-2 therapy on intrarenal

Foxp3+ Treg and kidney infiltrating CD4+ T cells in (NZBxNZW) F1 mouse model of lupus nephritis.
Materials and methods: (NZBxNZW) F1 mice with active nephritis were treated with recombinant IL-2 either over a short period of 24 h or over a long period of 20 days with PBS as control. Absolute numbers, phenotype and proliferation of kidney infiltrating CD4+ T cells were determined by flow cytometry.
Results: (NZBxNZW) F1 mice treated over a short term with IL-2 showed an enhanced proliferation of FoxP3+ Treg and increased numbers and frequency of CD4+Foxp3+ Treg compared to PBS treated control mice. On the other hand, long term IL-2 treatment did not result in a persistent expansion of the intrarenal Foxp3+ Treg population. Nevertheless, total numbers of kidney infiltrating CD4+ T cells were diminished and the CD4+ Tcon showed reduced signs of cellular activation.
Conclusions: Our data indicates that short term IL-2 treatment is able to expand the size of the intrarenal Treg pool. In contrast, long term IL-2 treatment decreases the numbers of kidney infiltrating CD4+ T cells. These results may in part explain the delay of disease progression induced by treatment with IL-2 and underline the important role of intrarenal Treg for the suppression of kidney disease in lupus mice. These results also provide additional rationales for an IL-2 based immunotherapy of human disease.

P1259

Lactobacillus rhamnosus KL-37 – derived, highly purified, exopolysaccharide (EPS) inhibits adjuvant effect of LPS on the development of CIA in DBA/1 mice

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Purpose/Objective: We have shown that crude exopolysaccharide (EPS), produced by *Lactobacillus rhamnosus KL-37* has immunomodulating activity both *in vitro* (cytokine production by mouse peritoneal macrophages) and *in vivo* (antigen-specific antibody production). The aim of present study was to examine the effect of highly purified EPS, free of LTA, DNA and bacterial proteins, on the onset of CIA induced in DBA/1 mice by collagen in the presence of bacterial adjuvant (LPS).
Materials and methods: To induce CIA DBA/1 male mice were given subcutaneous injection of chicken collagen type II (CII) emulsified in Complete Freud's Adjuvant (CFA). Twenty-one days later mice were boosted with CII in the presence of LPS (given intraperitoneally). EPS (or PBS as control) was given systemically (intravenously or intraperitoneally) or locally by subcutaneous injection every other day starting at the day of boost immunization. In some experiments EPS was given prior immunization. The development of CIA was evaluated by visual observation of joint inflammation and measurement of paw thickness. At the end of the experiment (day 42–45), hind paws were collected for histological evaluation of joint damage or MPO level in inflamed joints. Additionally, the level of proinflammatory cytokines (IL-6, TNF-alpha) and anti-CII antibodies were measured in serum by ELISA.
Results: Highly purified EPS, given to DBA/1 mice intravenously or intraperitoneally, ameliorated the onset of CIA. The incidence and severity of joint inflammation was reduced in mice receiving EPS during and after boost immunization (CII+LPS). Additionally, when given systemically, EPS reduced the level of anti-CII antibodies in serum. The incidence of CIA and clinical symptoms of joint inflammation were also ameliorated by subcutaneous injections of pure EPS although the level of anti-CII antibodies in serum was comparable to control mice (given PBS). The inhibiting effect of EPS on CIA development was not observed when EPS was given prior boost immunization with CII+LPS.
Conclusions: *Lactobacillus rhamnosus KL-37* derived EPS, devoid of other bacterial components (LTA, DNA, bacterial peptides), inhibits LPS, but not CFA, adjuvant effect on the development of CIA in DBA/1 mice.

P1260

Maternal systemic lupus erythematosus affects neonatal circulating plasminogen activator inhibitor-1 levels

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Purpose/Objective: During pregnancy changes in hemostasis takes place including a reduced fibrinolysis. Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by pronounced systemic inflammation and many patients have a disturbed fibrinolytic activity. Since pregnancy as such, is a condition with substantial impact on both inflammation and coagulation, women with SLE might be particularly exposed to pregnancy-associated complications. Therefore we have investigated fibrinolytic activity in pregnant women with SLE. In addition, fibrinolytic activity in cord blood of children to women with SLE or the closely related autoimmune disease anti-phospholipid syndrome (APS) was investigated, to elucidate if the disease of the mother affects fibrinolysis in the newborn child.
Materials and methods: To investigate the fibrinolytic activity we measured serum PAI-1 and PAI-2 concentrations with ELISA. Placental tissue was stained for PAI-1 and PAI-2 using immunohistochemistry.
Results: PAI-1 concentrations in serum from SLE patients were significantly higher during pregnancy and post partum compared to controls. Pregnant SLE patients also tended to have increased PAI-2 in serum compared to healthy women. However, no significant differences were found in placental PAI-1 or PAI-2 expression between SLE and controls. Finally, children to SLE and APS patients had the highest cord blood serum concentrations of PAI-1 when compared to children to healthy women.
Conclusions: PAI-1 and PAI-2 have been attributed to immune response modulation; PAI-1 has been proposed to enhance Th2 immune responses and PAI-2 is associated with suppression of Th1 immune responses. The pregnant SLE patients had increased *in vivo* concentrations of both PAI-1 and PAI-2, which might contribute to the proposed Th2 skewing in SLE patients. Finally, newborn children to women with SLE or APS seem to have a decreased fibrinolytic activity, which might have implications for these children during the neonatal period.

P1261

Membrane-bound and soluble BAFF expression by human rheumatoid fibroblast-like synoviocytes in response to TLR stimulation

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Purpose/Objective: B cell activating factors of TNF family (BAFF) is associated with the survival and maturation of B cells. BAFF is widely expressed in the rheumatoid arthritis (RA) synovium which is characterized by the presence of synovial niches of autoreactive B cells and

sustain *in situ* autoantibody production. Importantly, B cell niches remain functional in the RA-SCID model in the absence of recirculating cells, suggesting that autocrine mechanisms support ongoing B cell activation in the RA synovium. BAFF exerts its functional role both as a membrane bound protein and in soluble form. Here we investigated whether resident stromal cells in the RA synovium, synovial fibroblasts (RASF), are capable of producing either forms of BAFF and thus contribute to local B cell activation.

Materials and methods: mRNA BAFF in RASF stimulated with Toll-like receptors (TLR) 2, TLR3 and TLR4 ligands was assessed by quantitative Taqman PCR. RA dermal fibroblasts (RADF) and osteoarthritis SF (OASF) were used as controls. The cytoplasmic, membrane bound and/or soluble forms of BAFF were investigated by (1) Western blot using total and membrane-enriched protein extracts, (2) flow cytometry, (3) ELISA and (4) immunocytochemistry.

Results: *In vitro* stimulation of TLR3, and to a significantly lesser extent TLR4, but not TLR2 on RASF led to strong induction of BAFF mRNA. In response to TLR3, soluble BAFF was time-dependently released in the supernatant of RASF (~600 pg/ml) and, to a lesser extent, OASF and RADF. RASF constitutively expressed both cytoplasmic and membrane bound BAFF as demonstrated by Western blot, flow cytometry and immunocytochemistry which was upregulated upon TLR3 stimulation and was significantly increased as compared to RADF.

Conclusions: Here we provide conclusive evidence that SF in the RA synovium are a pivotal source of the B cell survival factor BAFF at both mRNA and protein level. In addition to their significant constitutive expression, RASF can further up-regulate cytoplasmic, membrane-bound and soluble BAFF in response to TLR3 stimulation. Overall, our data strongly support a fundamental role for RASF in sustaining functional B cell activation and antibody production in the inflamed RA synovium.

P1262

Modulation of rheumatoid adipose-derived mesenchymal stem cells function by adipocytokines

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Purpose/Objective: To investigate whether selected adipocytokines i.e. leptin, low (LMW) and high (HMW) molecular weight adiponectin isoforms and TNF affect indoleamine 2,3-dioxygenase (IDO) and hem oxygenase-1 (HO-1) mRNA expression and cytokines (IL-6, IL-8) secretion by rheumatoid adipose-derived mesenchymal stem cells (RA-ADSCs). To determine if conditioned media from RA-ADSCs treated with above mentioned adipocytokines, influence mRNA expression (APRIL, Cadherin-11, MMP-13), cytokine secretion (IL-6, IL-8, MMP-3) by FLS and FLS and PBMC apoptosis.

Materials and methods: Articular adipose tissue (AAT) and synovial membrane were obtained from rheumatoid arhritis (RA) patients during total knee joint replacement surgery. ADSCs were isolated and cultured with/without human recombinant leptin, TNF, adiponectin (LMW, HMW) and IFNγ. After 24 h, secretion of IL-6 and IL-8 and IDO, HO-1 mRNA expression was measured. Moreover, conditioned media from ADSC cultures were used to stimulate FLS (for 24 h) and PBMC from healthy donors (for 3 h). After stimulation, FLS and PBMC apoptosis was determined as well as gene expression and cytokine secretion in FLS. mRNA expression was assessed by Real Time PCR, cytokines concentration by ELISA and apoptosis by flow cytometry. The Wilcoxon signed-rank test was used for statistical analysis.

Results: Unstimulated RA-ADSCs did not express IDO mRNA. All applied stimuli triggered IDO expression, but with different potency. RA-ADSCs expressed HO-1 mRNA spontaneously and there were no significant differences after the treatment. IL-6 and IL-8 secretion were significantly up-regulated after HMW adiponectin ($P = 0.018$ and $P = 0.028$, respectively) and TNF treatment ($P = 0.018$). RA-ADSC-conditioned media exerted only slight effect: we did not observe any regularity in cytokines secretion by FLS, except IL-6 which was decreased after stimulation with media from HMW adiponectin-treated ADSCs ($P = 0.018$). mRNA expression for Cadherin-11, APRIL and MMP-13 stayed unchanged. No statistically significant differences in FLS and PBMC apoptosis after RA-ADSC-conditioned media treatment were observed.

Conclusions: Adipocytokines may influence RA-ADSCs function; however we did not prove it in all studied aspects. Further studies are needed to elucidate the hypothesis that the specific milieu of rheumatoid joint formed by pro-inflammatory cytokines and adipokines may affect properties of ADSCs from AAT.

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P1265

Over-expression of 5-alpha reductase in B cells from systemic lupus erythematosus patients suggests disease related difference in androgen activation

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Purpose/Objective: The prevalence of many autoimmune conditions is higher in women compared to men. These differences may be explained by the conflicting effects androgens and estrogens have on the immune system; androgens such as 5α-dihydrotestosterone (5α-DHT) have an anti-inflammatory effect, whereas estrogens such as 17β-estradiol enhance immune responses. Five-alpha reductase (SRD5A1) plays an important role in the regulation of androgen activity by converting testosterone into the more active 5α-DHT. Here, we have explored the expression of SRD5A1 in peripheral blood mononuclear cells from patients with SLE and in age and gender matched healthy controls.

Materials and methods: Cells were investigated with and without *in-vitro* stimulation using beads coated with anti-CD3 and CD28 antibodies. PBMC were labelled for SRD5A1 and cell populations were identified by expression of CD19, CD14, CD4, CD8 and CD45RO and analysed by flow cytometry.

Results: Both in healthy controls (HC) and in SLE patients T cell activation lead to an increase in SRD5A1 expression (HC $P = 0.002$, SLE $P \leq 0.0001$). Also, CD45RO+ T cells consistently showed a higher expression of 5-alpha reductase when compared to the CD45RO- subset.

We then investigated whether this mechanism is altered in autoimmune conditions by measuring the expression of SRD5A1 in various immune cells from SLE patients ($n = 30$) and healthy controls ($n = 16$) using flow cytometry. There was a significantly higher expression of SRD5A1 in B cells from SLE patients compared to healthy controls ($P = 0.005$).

Conclusions: So far, the data suggest that T cells up-regulate their capacity to activate testosterone to 5α-DHT upon activation and keep it stably up-regulated in the CD45RO+ subset. B cells from SLE patients are increasing their exposure to more potent androgens, which maybe an attempt to reduce B cell hyper-reactivity. As SRD5A1 inhibitors are used clinically to treat prostate cancer, it will be important to investigate the consequences of their use on the immune system.

P1268

Phenotypic and homeostatic abnormalities of Foxp3+ Treg in Systemic Lupus Erythematosus are associated with IL-2 deficiency

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Purpose/Objective: Systemic lupus erythematosus (SLE) is characterized by uncontrolled activation of T and B cells resulting in loss of tolerance towards self antigens. Regulatory T cells (Treg) are important players in the maintenance of self-tolerance. Phenotypic and quantitative abnormalities of Treg have been associated with human and murine SLE. Further, it was shown that SLE T cells have an impaired production of IL-2, which is the essential cytokine for Treg homeostasis. In order to substantiate a link between IL-2 deficiency and Treg abnormalities in SLE we investigated the phenotype and homeostasis of CD4+Foxp3+ Treg and CD4+Foxp3- conventional T cells (Tcon) in view of abnormalities associated with an IL-2 deficiency.
Materials and methods: Phenotype, frequency and proliferative status of Treg and Tcon subsets were analyzed in peripheral blood from 90 SLE patients and 40 healthy donors by flow-cytometry. Disease activity was determined according to the SLE activity index (SLEDAI). Two-tailed Mann−Whitney *U*-test was used for statistical analysis between SLE and control groups, Spearman's rank coefficient was used to calculate correlations with disease activity.
Results: The frequency of Foxp3+ Treg is significantly increased in SLE patients compared to healthy controls ($P = 0.015$). However, their phenotype is considerably altered: We found a significantly reduced frequency of CD25 expressing cells among Foxp3+ Treg in SLE ($P = 0.0001$) and the frequency of cells with a memory phenotype was increased ($P = 0.003$). Analysis of Ki67 expression revealed an increased percentage of proliferating Treg and Tcon, which correlated with disease activity ($r = 0.36$, $P = 0.012$ and $r = 0.46$, $P = 0.001$ resp.). The ratio of proliferating Treg versus proliferating Tcon, however, was significantly reduced in SLE compared to controls ($P = 0.0001$) and inversely correlated with disease activity ($r = -0.33$, $P = 0.02$).
Conclusions: Although the frequency of Foxp3+ Treg is increased in SLE patients, their loss of CD25 expression and their shift to the memory compartment together with the homeostatic imbalance between Treg and Tcon indicate a profound disorder in Treg biology in SLE. These phenotypic and homeostatic abnormalities of SLE Treg are very similar to those observed in IL-$2^{-/-}$ and lupus-prone mice, suggesting an IL-2 deprivation of Treg also in human SLE.

P1271

Positive double-stranded DNA antibodies: it is not always lupus

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Purpose/Objective: Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease with relevant clinical and immunological manifestations in which double-stranded DNA (anti-dsDNA) antibodies have been considered the serological hallmark. About 75−95% of patients with active and non-active disease produce anti-dsDNA antibodies. The aim of this study is to evaluate different assays for anti-dsDNA antibodies detection.
Materials and methods: Retrospective study with seventy two sera from Germans Trias i Pujol Hospital. All sera were positive for anti-nuclear antibodies (ANA, homogeneous pattern with a title higher than 1:80). Each serum sample was analyzed with QUANTA Lite®dsDNA ELISA (test A) and QUANTA Lite®dsDNA SC ELISA (test B) in order to determine the presence of anti-dsDNA antibodies and with QUANTA Lite™Chromatin ELISA (test C) and Anti-dsDNA-NcX ELISA (test D) to detect antibodies against the dsDNA-nucleosomes complex. All corresponding clinical notes were reviewed.
Results: Fifty four sera with test A and sixty sera with test B were positive for anti-dsDNA antibodies test showing a sensibility of 68.4% and 80.7% respectively but very low specificity (≤ 6.6%). In relation to antibodies anti-dsDNA-nucleosomes complex: sixty five sera with test C and fifty three sera with test D were positive with a sensibility of 91.2% and 82.4% respectively and a specificity of 13.3% and 60% respectively. False positive Results: (patients without SLE but with a positive anti-dsDNA determination) were less common in test detecting anti-dsDNA-nucleosomes antibodies ($n = 13$ (18%) and $n = 6$ (8.3%), respectively), than those detecting anti-dsDNA antibodies ($n = 15$ (18%) and $n = 14$ (20%), respectively). False positive results were mostly from patients with infection (HBV, HCV and HIV).
Conclusions: The determination of antibodies against the dsDNA-nucleosomes complex displays higher specificity for SLE than the determination of anti-dsDNA antibodies alone. The selection of the antigen should be taken into account for the diagnosis and follow up of SLE patients.

P1272

Protective role of MAP kinase phosphatase 2 (MKP-2) in a model of collagen antibody induced Arthritis

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Purpose/Objective: MAP kinase phosphatase 2 (MKP-2) is a nuclear phosphatase which negatively regulates the MAP kinase signalling pathway by dephosphorylating MAP kinases ERK, JNK and to a lesser extend p38. The involvement of the MAPK pathway in the development of Arthritis has been previously shown and a protective role has been established for the related phosphatase MKP-1 in macrophages (Salojin *et al.* 2006). MKP-1 and MKP-2 share many properties and consequently we investigated the role of MKP-2 in Arthritis development.
Materials and methods: We recently generated MKP-2 deficient mice and utilised these to study the role of MKP-2 in the development of collagen antibody induced arthritis (CAIA). In addition we measured neutrophil infiltration into the joints using an *in vivo* imaging system (IVIS), myeloperoxidase expression and further analysed cytokine and chemokine levels in joints and serum of mice.
Results: We found that MKP-2 deficient mice were particularly responsive to collagen antibody induced arthritis and developed severe signs of disease marked by earlier onset, stronger and prolonged symptoms (weight loss, swelling of joints, especially front paws and ankles) compared with their wild type counterparts. This was accompanied with a drastically enhanced infiltration of neutrophils into the joint cavities, increased pathology of the joint and higher levels of monocyte chemotactic protein MCP-1 in the joints and the serum.
Conclusions: We show that MKP-2 plays a protective role in arthritis, as the absence of this molecule results in severe disease.

P1273

Regulatory T cell function is influenced and partly restored by commonly used biologicals in rheumatoid arthritis (RA)

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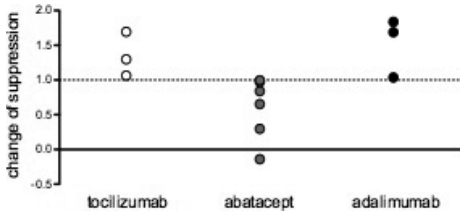
Purpose/Objective: Rheumatoid arthritis (RA) therapy includes a variety of biologicals that either block different pro-inflammatory

cytokines such as IL-1 (anakinra), TNF (etanercept, infliximab, adalimumab) or IL-6 (tocilizumab), modulate the adaptive immune system by depleting B cells (rituximab) or interfere with T cell activation and antigen presentation (abatacept). Regulatory T cells (Treg) are a crucial T cell subset in controlling autoimmune disorders and inflammatory responses in general. Here, we aim to understand the effect of three different biologicals, namely adalimumab, tocilizumab and abatacept on the functionality of Treg.

Materials and methods: Peripheral blood (PB) of RA patients was collected at baseline and after 3 month of abatacept ($n = 8$) or tocilizumab ($n = 10$) therapy. Specific T cell and Treg marker were determined *ex vivo* by multi-parameter flow cytometry. In addition, adalimumab, tocilizumab or abatacept was added to *in vitro* co-culture assays to assess the influence on synovial-fluid derived Treg and T effector cells (T eff) from chronic RA patients ($n = 12$).

Results: RA patients treated with abatacept showed a diminution of Treg-associated marker such as FOXP3 ($p \leq 0.05$), Helios ($p \leq 0.05$) and CD39 ($p \leq 0.05$). Tocilizumab therapy increased CD25high T cells ($p \leq 0.05$), but did not change FOXP3 frequency. Functional studies of synovial Treg with added adalimumab or tocilizumab showed an enhancement of their suppressive capacity when compared to control co-cultures. However, no beneficial effect on Treg suppressive capacity was seen when functional studies were performed in the presence of abatacept. Moreover, adalimumab and abatacept but not tocilizumab reduced the proliferative capacity of T eff cells when administrated *in vitro*.



Conclusions: All investigated biologicals do influence Treg function, but by different means. Abatacept did not increase Treg suppressive capacity, most likely due to its dual effect on T eff and Treg. Adalimumab increased suppression by its profound effect on T eff while tocilizumab also enhanced suppression but probably by affecting antigen-presenting cells in the culture. These data emphasize the need to understand Treg function in a context, which also takes into account the inflammatory milieu. Hence, many therapies can promote Treg function without directly interacting with this cell subset.

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Role of CCR2 expression in neutrophils on development o arthritis

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Purpose/Objective: Rheumatoid Arthritis (RA) is an autoimmune disease characterized by articular pain and progressive bone damage. These disease features have been associated with neutrophil infiltration on articular cavity. Blood neutrophils (PMN) trafficking during inflammation is a complex process with participation of several types of chemotactic factors. In general, PMN interact directly with CXC chemokines, as CXCL2 and monocytes and lymphocytes interact predominantly with CC chemokines (CCLs). However, our group showed that PMN from mice and patients with sepsis express a CCL receptor type 2 (CCR2). In sepsis, CCR2 expression has an essential role in neutrophil tissue infiltration. Here, we investigate the role of CCR2 on neutrophil infiltration on antigen-induced arthritis (AIA).

Materials and methods: PMN from healthy or RA patients, or from AIA mice were purified and used to chemotaxis assay and evaluation of CCR2 expression by qRT-PCR and immunofluorescence. AIA was induced in wild-type (WT) and CCR2 deficient mice ($CCR2^{-/-}$) 21 days after first mice sensitization by intra-articular injection (i.a.i) of antigen (mBSA) or CCL2. Some mice were also treated with CCR2 antagonist (RS504393) before challenges. PMN migration was assessed 7 h after i.a.i. articular hyperalgesia was evaluated at this same time using an electronic version of von Frey test. *In vitro* PMN chemotaxis in response to CXCL2 or CCL2 was performed in a Boyden microchamber. All experiments were approved by Human Ethics Committee (2981/2009) and Animal Ethics Committee (nordm; 181/2008) from HCFMRP/USP.

Results: PMN from RA patients and from arthritic mice express CCR2 and migrates to CCL2. Furthermore, CCL2 was able to induce PMN intra-articular infiltration in immunized mice. PMN recruitment to joints and articular hyperalgesia was significantly reduced in $CCR2^{-/-}$ mice or by treatment with RS504393. To ensure that CCR2 were directly responsible of PMN infiltration to the joint cavity we performed the adoptive transference of WT PMN from immunized mice to $CCR2^{-/-}$ immunized mice. This transference restored arthritis phenotype (PMN infiltration and articular hypernociception) to $CCR2^{-/-}$ mice after mBSA challenge.

Conclusions: Our findings suggest that CCL2-CCR2 chemotaxis pathway is involved directly in the detrimental infiltration of PMN to the joints in experimental arthritis.

P1275
Selective infiltration of CD6-CD56bright NK cells into the synovial fluids of rheumatoid arthritis and osteoarthrosis patients

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Purpose/Objective: Natural killer (NK) cells are part of the innate immune system and respond rapidly to a variety of stimuli via cytolytic activity and cytokine secretion. In the periphery, human NK cells can be divided into two major subsets defined as $CD16^+$ CD56dim and CD16-CD56bright NK cells. In this study, we wanted to demonstrate whether NK cells characterized by a clearly defined phenotype participate in the inflammatory process that is driving inflammation in rheumatoid arthritis but absent in osteo arthritis.

Materials and methods: Peripheral blood and synovial fluid of RA and OA patients was analysed by flow cytometry in order to define the distribution of NK cell subsets in this compartment, in addition, the microenvironment in plasma versus SF (synovial fluid) was analyzed by mutliplex based protein arrays for 50 cytokines, chemokines and growth factors. SF ofRheumatoid arthritis (RA) patients: $n = 33$ and Osteoarthritis (OA) patients: $n = 36$.

Results: Amongst all tested markers, the absence of CD6 represented the strongest correlation regarding NK cell infiltration, independently

from their CD16 expression. One reason for this reversed NK cell distribution could be the selective recruitment of CD56bright NK cells by chemokines. As shown in other publications, CD56bright NK cells express CXCR3 and CCR5 at higher levels whereas CD56dim NK cells express more CXCR1. Therefore the most important cytokines, chemokines and growth factors in synovial fluids and sera were determined by the Luminex-based multiplex method. As expected for CD56bright NK cells, two ligands for CXCR3 i.e. CXCL10 (IP-10) and CXCL9 (MIG) where found in significantly high concentrations in the SF of RA and OA patients compared to autologous sera. The same is true for the CCR5 ligand CCL4 (MIP-1b), however CCL5 (Rantes) was significantly decreased in the SF of RA and OA patients compared to sera. The CXCR1 ligands CXCL8 and CXCL1 are also present at significantly high concentrations while CXCL5 (ENA-78) is significantly reduced in SF compared to sera of both patient cohorts. Therefore the lack of the $CD16^+$ CD56dim NK cell infiltration is not due to the absence of their recruiting chemokines.

Conclusions: Our results indicate that CD6 is differentially expressed on NK cell subsets and represents a novel NK cell marker for infiltration into synovial fluid. Furthermore, the infiltration of CD6-CD56bright NK cells seems to be supported by high concentrations of the ligands CXCL10 (IP-10) and CXCL9 (MIG).

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Serum and urinary IL-18 levels are associated with SLE and lupus nephritis activity

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Purpose/Objective: IL-18, a proinflammatory cytokine released predominantly by antigen presenting cells, is elevated in association with SLE disease activity; and its glomerular expression is increased in lupus nephritis. The purpose of this study was toexamine the association of serum IL-18 with SLE disease activity and urinary IL-18 with lupus nephritis.

Materials and methods: SLE patients and healthy controls were recruited in accordance with institutional review board guidelines. SLE patients were from our longitudinal study cohort where demographics, disease manifestations, treatment regimes, disease activity indices (SLAM-R and SLEDAI) and SLE damage index (SDI) are prospectively recorded. Renal disease activity was defined as a score of more than zero for renal related criteria in SLEDAI (range 0−16). Serum and urine IL-18 levels (in pg/ml) were determined by ELISA. The correlation of serum IL -18 with disease activity, anti-dsDNA antibody (ab), C3, C4 and CRP levels, and disease manifestations was analyzed.

Results: Levels of serum IL-18 were significantly higher in SLE patients ($n = 891$) than normals ($n = 50$); 311.1 (IQR: 0−969.7) versus 159.6 (IQR: 86.0−217.3); $P = 0.03$ respectively. Serum IL-18 levels positively correlated with SLAM-R [inactive: 330.6 (IQR: 0−938.5), mild: 325.6 (IQR: 0−1068.7), moderate: 500.5 (IQR: 175.0−573.6), and severe 1620.7 (IQR: 243.9−2395.5), $P = 0.0055$], and median levels were higher in patients with active neurological [305.7 (IQR: 0−964.1) versus 574.5 (IQR: 186.9−1439.0); $P = 0.040$] and mucocutaneous [279.1 (IQR: 0−954.1) versus 449.9 (IQR: 0−1136.9); $P = 0.036$] manifestations. Significant correlation was found with ESR and CRP but not anti-dsDNAab or complement levels. Detectable urinary IL18 was found in 163 of 246 SLE patients, median 24.38, and inter-quartile range: 15.9−46.54, range: 10.16−550.6, mea $n = 42.40 \pm 58.50$, with a markedly skewed distribution. Healthy controls ($n = 21$) had lower urinary IL-18; mea $n = 29.41$, SD 21.68, SEM 4.73. A significant difference ($P = 0.015$) was found between different renal SLEDAI groups (modified Kruskal-Wallis tests for Trend across Ordered Groups), and a clear difference found also between renal SLEDAI = 0 compared to renal SLEDAI = 4 (Wilcoxon rank sum test, $P = 0.029$). However the number of patients with higher grades of renal activity was too few for meaningful comparison.

Conclusions: Serum IL-18 levels are increased in SLE, particularly in those with active neurological and mucocutaneous manifestations, and correlate with disease activity. Urine IL-18 levels significantly correlated with renal lupus disease activity. Further studies are warranted to determine the usefulness of this cytokine in assessing activity of lupus nephritis.

P1277

Severe defects of complement regulator CD46-mediated IL-10 producing regulatory T cells in patients with systemic lupus erythematosus

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Purpose/Objective: SLE is a complex autoimmune disease characterised by the breakdown of tolerance. Although the pathogenesis of SLE is not entirely understood, evidence suggests a strong genetic component. We have identified the TNF superfamily member *OX40L* as a candidate risk gene. It has been shown that OX40L completely inhibits the generation of IL-10-producing $CD4^+$ T cells resembling an adaptive regulatory T cell type (Tr1). IL-10 is key to the maintenance of peripheral tolerance. We therefore became interested in the examination of these $CD4^+$ T effector cells in SLE.

Materials and methods: Thirty seven SLE patients and 20 healthy controls were recruited for this study. Purified $CD4^+$ T cells were stimulated with antibodies to CD3 and CD46 in the presence of IL-2 and cytokine production was measured using the human IFN-γ or IL-10 Cytokine Secretion Assay kit (Miltenyi). Suppression assays were performed by monitoring cell proliferation of freshly isolated CD3/CD28 stimulated T cells cultured in the supernatant of CD3/CD46 stimulated cells.

Results: We found a striking difference in the cytokine profile of CD3/CD46 stimulated T cells from SLE cases compared to healthy controls. T cells in the majority of patients lacked an $IL\text{-}10^+$ population, produced up to 10 times more IFN-γ compared to healthy controls ($P < 0.0001$) and switching into an $IFN\text{-}\gamma^+/IL\text{-}10^+$ regulatory phase was defective. These results were consistent in a subset of patients when repeated 1 month later. Furthermore we showed that the IL-10 dependent suppressive activity of Tr1 cells was impaired in SLE. A neutralizing antibody was used to determine the contribution of OX40L to the development of Tr1 cells. We could not replicate the previously shown involvement of OX40L.

Conclusions: We have demonstrated that the CD46-mediated production of IL-10 producing effector T cells is dysregulated in SLE, a novel mechanism that might contribute to the development of the disease. Impaired Tr1 cell function is also observed in other autoimmune diseases like multiple sclerosis and rheumatoid arthritis. Together with our results it seems very likely that a defect in the CD46-mediated pathway is a common autoimmune phenotype. Thus understanding the precise signalling pathway in T cells associated with the production of IL-10 may lead to promising targets for the development of novel therapies for the treatment of autoimmune diseases.

P1279

Succinate enhances experimental arthritis

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Purpose/Objective: It has been shown that succinate, a citric acid-cycle intermediate, is released in the extracellular space upon cellular energy supply-demand imbalance or cell damage and can act as a mediator signaling through the G protein coupled receptor-91 (GPR91). Interestingly, activation of GPR91 on dendritic cells (DCs) by succinate enhances DC-mediated T cell activation and cytokine production. DCs play a key role in inducing and perpetuating the immune response in autoimmune diseases. The aim of present study was evaluate the effect of succinate on the development of experimental arthritis.
Materials and methods: Arthritis was induced by immunization of male C57BL/6 mice with methylated bovine serum albumin (mBSA) and completes Freund's adjuvant (CFA). Some mice received 2.5 mg of succinate in the antigen emulsion. Twenty-one days later, arthritis was triggered by intra-articular mBSA injection and articular hyperalgesia; leukocyte infiltration and cartilage proteoglycan loss was determined. Titers of serum anti-mBSA IgG and expression of IL-17/IFN by draining lymph node lymphocytes were also determined. All experiments were performed in accordance with protocols approved by the institutional Ethics Committee (protocol 146/2011).
Results: mBSA challenge into the femurtibial joint induced articular hyperalgesia and leukocyte infiltration. The inclusion of succinate in the antigen emulsion enhanced articular hyperalgesia ($t_8 = 4.48$, $P < 0.05$) and increased leukocyte infiltration in the joint cavity ($t_7 = 1.75$, $P < 0.05$) compared to control-immunized group. Serum from succinate-treated mice contained significantly higher concentrations of mBSA-specific IgG ($t_7 = 2.54$; $P < 0.05$). Moreover, succinate-treated mice had higher cartilage proteoglycan loss ($t_7 = 2.4$, $P < 0.05$). The expression of IL-17 lymphocytes was higher in succinate-treated mice ($t_7 = 3.16$, $P < 0.05$) while there are not change in lymphocytes producing IFN ($t_7 = 0.31$, $P < 0.05$).
Conclusions: We show here that succinate given together with the antigen emulsion potentiates specific immune response, enhancing articular inflammation during antigen-induced arthritis. Although the exact mechanisms by which succinate mediates its effects was not elucidated yet, our data suggest that the release of succinate could be an important component for the self-tolerance breaks down in autoimmunity.

P1280

Targeting the autoreactive memory using a new therapy combining plasma cell depletion with inhibition of activation and differentiation of B cells

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Purpose/Objective: In Systemic lupus erythematous (SLE) autoreactive plasma cells (PC) producing autoantibodies can survive in dedicated niches where they are resistant to immunosuppression and cytotoxic drugs. Therefore these cells represent a promising target for new therapies. It has been shown that the proteasome inhibitor bortezomib is able to deplete PC in NZB/W mice leading to an amelioration of the renal disease. However, it is likely that after PC-depletion new autoreactive PC can be generated from B cells and contribute to the maintenance of phatogenic memory. Therefore in this study we tested a new treatment approach aimed at depleting PC and, at the same time, preventing the generation of new autoreactive PC that results from B cell-hyperreactivity. We combined bortezomib with an anti-CD20 antibody or with the immunosuppressive drug cyclophosphamide (CY) and analyzed the PC-B cells dynamics in bone marrow (BM) and spleen of NZB/W mice.
Materials and methods: Mice were injected i.v. or not with 250 μg of a murine anti-CD20 antibody or 35 mg/kg body weight (BW) CY at various time intervals. All the mice where then injected twice whit 0.75 mg/kg BW bortezomib. The mice were sacrificed at different time points after the treatment, and PC and B cells subsets were characterized by FACS and ELISPOT.
Results: The selective depletion of PC using bortezomib without depletion of their precursor's leads only to transient effects as production of newly formed PC continues throughout life and the PC-niches are refilled quickly. Therefore, we treated NZB/W mice with bortezomib in combination with a murine anti-CD20 antibody or CY. The groups treated with the combination therapies showed a heterogeneous grade of B cell depletion and a slower repopulation of the PC pool. However, the supply of newly generated autoreactive PC from hyperreactive-B cells was not completely suppressed.
Conclusions: In this study, we observed that the PC niches in spleen and BM were refilled within few days after bortezomib mediated depletion, which is caused by persisting B cell hyperactivity. Due to the observed resistance of NZB/W mice to B cell depletion, neither anti-CD20 nor CY therapies were able to completely prevent the differentiation of autoreactive B cells in PC after depletion. Therefore, for sustained depletion effects and thereby sustained remission in patients the combined depletion of autoreactive PC and their precursor cells (i.e. B cells) is necessary.

P1282

The role of miR-155 in monocyte migration in Rheumatoid arthritis

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Purpose/Objective: Rheumatoid arthritis (RA) is a chronic autoimmune disease that leads to joint destruction. The recruitment of effectors cells, including monocytes to the joint space is an important step in RA progression and is mediated by chemokines (Ch) and their receptors (ChR). MicroRNAs are a recently discovered class of post-transcriptional regulators. Many members of the miR family are implicated in the regulation of cell movement and migration. Our previous study showed miR-155 is upregulated in RA synovial fluid (SF) monocytes suggesting that this miR may be involved in activation of these cells, including their migration into joint space. We hypothesized that miR-155 could regulates migration of monocytes in RA by modulating the expression of the chemokine and chemokine receptor system.
Materials and methods: Peripheral blood (PB) $CD14^+$ cells from healthy controls (HC) and RA patients were transfected with miR-155 mimic or scramble mimic using N-TER nanoparticles and cultured for 48 h. TaQman Low Density Array and multiplex assay was used to evaluate ChR expression and Ch production, respectively. Similar analysis was carried out on bone marrow monocytes (BMM) from miR-$155^{-/-}$ and WT mice. In addition, absolute copy numbers of miR-155 transcripts in PB and SF $CD14^+$ of RA and HC were assessed by QPCR.
Results: PB and SF monocytes in RA patients showed higher copy number of miR-155 compared to HC. Overexpression of miR-155 in HC and RA monocytes did not affect the production of CCL2, CCL7,

CCL21, CXCL5, CXCL8, CXCL7, CXCL10 and CX3CL1. In contrast, overexpression of miR-155 induced the production of chemokines such as CCL4, CCL5 and CCL22 in RA monocytes and CCL3 in both RA and HC. Analysis of chemokine receptors in BMM of miR-155$^{-/-}$ and WT mice revealed significantly higher levels of CCR1, CCR2, CCR5 and CXCR4 in miR-155 deficient cells suggesting that miR-155 can act as a negative regulator of these receptors in homeostatic state. As expected, TLR-4 ligand significantly suppressed expression of these receptors in both WT and miR-155$^{-/-}$ cells. Analysis of 3'UTRs of Ch/ChR (TargetScan) suggests that miR-155 is likely interfering with signaling pathways implicated in Ch/ChR system expression.
Conclusions: Deregulation of miR-155 in RA monocytes can contribute to the production of pro-inflammatory chemokines by these cells and to their accumulation at sites of inflammation.

P1283
The role of TNFa in the maintenance of joint inflammation in the CAIA model of Rheumatoid Arthritis

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Purpose/Objective: Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by synovitis, systemic inflammation and production of autoantibodies. The introduction of treatment with biologics, such as those targeting TNFα, have revolutionised the treatment of RA. Here we investigate the role of TNFα in the collagen antibody induced arthritis (CAIA) model of inflammatory arthritis.
Materials and methods: Male Balb/C mice were administered with 4 mg of anti-collagen II mAb cocktail on day 0. On day 1 the mice are administered with 25 μg LPS. Animals were weighed and scored for clinical signs of arthritis (swelling) daily. Using MSD multiplex ELISAs, cytokines, including TNFα, levels were measured in the paws and serum in the CAIA model at time points prior to and post LPS administration. To investigate TNFα inhibition mice were treated with either a anti-TNF Ab, 100 mg/kg i.v. 1× weekly, etanercept 30 mg/kg q.a.d. s.c., or anti-p55 Ab 30 mg/kg i.v. 2× weekly. To determine a dose response and to establish a time frame of efficacy the anti-TNF Ab was administered 1 h prior to Ab cocktail at 100 mg/kg, 6 h post LPS at 100, 3 and 0.01 mg/kg, and 24 h post LPS at 100 mg/kg.
Results: In the CAIA model TNFα levels were at their highest around 1 h post LPS in the serum and 2.5 h post LPS in the paws. Serum TNFα levels fall to baseline 24 h post LPS and were not detected in the paw from 6 h post. Treatment with Anti-TNF, etanercept and anti-p55 in this model significantly inhibit clinical scores by 100%, 99.5% and 96.5% respectively. Dosing prior to LPS and 6 h post LPS significantly inhibited clinical score. A dose dependent inhibition was observed with administration of anti-TNF, 100 mg/kg and 3 mg/kg, significantly inhibiting clinical score 98% and 75.8% respectively and 0.01 mg/kg showing a 17.8% non significant increase. Dosing 24 h post LPS with 100 mg/kg of anti-TNF did not significantly reduce clinical scores.
Conclusions: Through analysis of TNFα levels in the CAIA model and the administration of anti-TNF Abs we have determined that TNFα is essential in initiating disease but its role is less critical 24hrs post LPS and as such in the progression of inflammation in the paws. It is possible that other cytokines such as IL-1β are key in driving the inflammatory process in the latter stages.

P1284
TLR9-independent and immune complex-independent interferon-alpha production by neutrophils upon netosis in response to circulating chromatin

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Purpose/Objective: Chromatin represents a major autoantigen in systemic lupus erythematosus (SLE). Interferon (IFN)-α plays an important role in lupus development. Plasmacytoid dendritic cells (pDC) are believed to be the main producers of IFN-α in SLE, although they represent a minor cell population. In contrast, neutrophils represent 50% of total blood leukocytes and are activated in SLE, especially by chromatin. Toll-like receptor (TLR) 9 recognizes certain forms of DNA but its role in SLE is still not elucidated. We therefore sought to determine the cellular source of IFN-α as well as the natural stimuli in SLE and the impact of TLR9.
Materials and methods: Chromatin was purified from calf thymus. PBMC and neutrophils were isolated from healthy individuals, SLE and rheumatoid arthritis patients. Mouse neutrophils were purified from the bone marrow. Cells were activated with different stimuli. Cell activation and IFN-α production/secretion was verified by flow cytometry and ELISA. Gene expression was analyzed by qPCR. Neutrophil extracellular trap (NET) induction was estimated by confocal microscopy.
Results: IFN-α secretion by neutrophils was observed with steady-state and not pro-inflammatory neutrophils, from both healthy donors and patients whereas pDC were less efficient. Neutrophil-derived IFN-α was detected in response to free chromatin, and not chromatin-containing immune complexes, as well as TLR9 agonists. Nucleosome induced IFN-α production by neutrophils was associated with IL-8 secretion, CD66b up-regulation, ROS production and NET formation. Neutrophil priming is not required. PBMC sustain IFN-α secretion by chromatin-activated neutrophils in co-cultures. Importantly, chromatin-induced IFN-α secretion occurs independently of TLR9. Finally, chromatin increases gene expression levels of IFN-α and several DNA sensors, e.g. AIM2 and STING.
Conclusions: Neutrophils represent a major source of IFN-α. This is the first report showing both that steady-state neutrophils can secrete IFN-α and identifying a natural lupus stimulus involved. A key event is thus the presence of increased concentrations of circulating nucleosomes in SLE patients. Chromatin-activated neutrophils may secrete IFN-α early during SLE development, before immune complexes are produced. The generation of NET and the expression of genes involved in the recognition of DNA may strengthen pDC activation and DNA-mediated activation.

P1286

ZNF334 – a new molecular marker of rheumatoid arthritis – sensitive to proinflammatory cytokines

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Purpose/Objective: We have shown before thatthe expression of ZNF334 gene, coding for a new zinc finger protein, is extremely reduced in the $CD4^+$ lymphocytes of RA patients regardless their age and sex and thus can be considered a new molecular marker of the disease (Soroczynska-Cybula *et al. Immunology* 2011, 132(1):78–86). Based on the promoter sequence of the gene we speculated that it might be regulated by TNF, similarly to regulation of CD28 and Klotho genes described earlier. Here we have tested that hypothesis.

Materials and methods: The objectives were achieved by studying the *in vitro*influence of TNF, IL-1 and IL-6 on the levels of expression (by real-time PCR) and contents ofZNF334 protein (by Western blot) in the $CD4^+$ cells of healthy volunteers, both *ex vivo* and after 24–120 h of anti-CD3 stimulation *in vitro*.

Results: We have confirmed that TNF modifies the expression of ZNF334 in the CD4+ cells *ex vivo*; however, the effect varied for different individuals and reduction of expression was seen only for those cell samples that initially exhibited high transcriptional activity of the gene, while for those exhibiting initially very low expression, some increase in the transcriptive activity of the gene could be shown. On the other hand, both IL-1 and IL-6 had variable, but negligible-to-increasing effect on ZNF334 expression, never showing an inhibitory effect. The expression of ZNF334 in $CD4^+$ lymphocytes isolated after various periods of anti-CD3 stimulation was also variable, but generally increased with longer culture times, both with and without TNF treatment. Finally, incubation with TNF significantly reduced the amounts of two isoforms of ZNF334 protein in the $CD4^+$ cells of healthy volunteers.

Conclusions: Our Results: , albeit preliminary, seem to confirm the regulatory effect of TNF (and possibly also IL-1 and IL-6) on the expression and protein levels of ZNF334 zinc finger protein in healthy helper T lymphocytes and to stress that this effect varies in different individuals, at least regarding the gene expression. It is too early now to speculate what might be the meaning of our observations (other than a general remark that it might regulate the expression of as yet unknown gene(s) and that its regulatory properties would be modified in RA lymphocytes), as no specific functions for ZNF334 had been reported yet.

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Poster Session: Sepsis

P1288
CD44-deficiency delays LPS-induced acute renal injury

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Purpose/Objective: Sepsis is a progressive syndrome associated with a disseminated inflammatory response that leads to multiorgan failure, including acute kidney injury (AKI), which occurs in 20−50% of septic patients. CD44 is a cell-surface transmembrane glycoprotein expressed on a variety of cell types such as haematopoietic, epithelial, endothelial cells and it is upregulated in the renal cortex upon injury. CD44 plays a role in proinflammatory cytokines production, leukocytes migration and adhesion, and TLRs-signalling regulation and it forms a complex with TLR-4 and MD-2.
Materials and methods: In order to understand the role of CD44 in sepsis-induced acute kidney injury, we compared Wt and CD44 KO mice (n = 8/group), which received an intraperitoneal injection of 10 μg/g body weight of LPS. 2, 4, and 24 h after LPS injection, mice were sacrificed and both kidneys and blood were collected for analysis of kidney function, damage and inflammatory state.
Results: At 2 and 4 h after LPS injection, the plasma and renal levels of the pro-inflammatory cytokines MCP-1, TNF-α, IL-1β were lower in CD44 KO mice in respect to the Wt, and the opposite was found for the anti-inflammatory cytokines IL-10 and HGF. This was associated with decreased T cells influx in the CD44 KO kidneys. Furthermore, TLR4 gene expression in the CD44 KO kidneys was induced later and to a lesser extent than in the Wt; while the mRNA expression of the protective HO-1 (heme oxygenase-1) was higher in the CD44 KO kidneys than in the Wt. This could explain the cytokines pattern of the CD44 KO mice, as TLR4 signaling leads to secretion of pro-inflammatory molecules and HO-1 controls the balance pro-/anti-inflammatory cytokines. LPS-induced iNOS expression in renal tubules is parallel to the time course of capillary dysfunction and the iNOS levels found in the CD44 KO renal tissue remained low at 2 and 4 h compared to the Wt. This was associated with preserved renal function and decreased apoptosis rate of tubular and interstitial cells in the CD44 KO kidneys compared to Wt at 2 and 4 h.
Conclusions: In conclusion, these data suggest that the lack of CD44 protects against sepsis-induced AKI by impairing the leukocytes influx in the kidney parenchyma, the production of nitric oxide, the pro-inflammatory cytokines secretion via HO-1 induction and delayed TLR4 expression and therefore maintaining a better renal function.

P1289
Chitohexaose activates macrophages by alternate pathway through TLR4 and blocks Endotoxemia

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Purpose/Objective: Sepsis is one of the leading causes of death contributing to half of mortality in intensive care units across the world. LPS present in the cell wall of gm-negative bacteria signals through TLR4 and activates macrophages to produce inflammatory mediators such as TNF-α, IL-1β, IL-6 etc, is a critical factor inducing endotoxemia and shock in most cases of sepsis. This study aimed to develop an antagonist to inhibit the LPS mediated inflammation and to test its efficacy in protection from endotoxic shock.
Materials and methods: Preparation of filarial extracts and labelling Bone marrow cells culture Mouse Model of Endotoxemia Isolation of human PBMCs and *in vitro* culture Quantification of Cytokines Flow Cytometry, Nitrite analysis and Arginase assay Quantitative real timePCR analysis.
Results: In this study we report that a filarial glycoprotein (AgW) binds to TLR4 of human monocytes and murine macrophages through a carbohydrate residue (i.e. chitohexaose) present in AgW. LPS mediated induction of TNF-α, IL-1β and IL-6 by macrophages/monocytes was effectively inhibited both *in vitro* and *in vivo* by AgW as well as chitohexaose. Inhibition of LPS mediated inflammation by chitohexaose appeared to be a consequence of activation of macrophages into alternate pathway by the carbohydrate. Intraperitoneal administration of chitohexaose protected mice from lethal endotoxic shock. More crucially, therapeutic administration of chitohexaose after 6, 24 or 48 h of onset of endotoxemia also protected mice from lethal endotoxic shock and death. Ex-vivo analysis of monocytes filarial parasite infected humans revealed significantly lower numbers of inflammatory monocytes in comparison to endemic controls. Futher monocytes of filarial infected individuals expressed canonical markers of alternate activation were refractory to inflammatory response when stimulated with LPS. The significance of this finding in the context of Hygiene Hypothesis will be discussed.
Conclusions: We demonstrate thatchitohexaose delicately balances the storm of inflammation induced by LPS while concurrently activating macrophages into a non inflammatory alternate pathway through TLR4. Administration of chitohexaose protected mice from endotoxemia prophylactically as well as therapeutically. The study also offered evidence for induction of two diverse activation pathways of macrophages through a single receptor, TLR4.

P1290
Epigenetic profile in lipopolysaccharide-stimulate macrophages

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Purpose/Objective: Sepsis remains a clinical challenge for the intensive care units. However, it is known that the tolerance mechanism using low doses of lipopolysaccharide (LPS) reduces the expression of proinflammatory genes and involves epigenetic regulation. The chromatin openness is regulated by Histone Acetyltransferases (HATs) and these enzymes could be modulated by Nitric Oxide (NO) interaction. In the present work, we demonstrate the pathway of tolerance to LPS from HAT activity and level of histone openness to production of cytokines as well as the influence of NO inhibition.
Materials and methods: THP1 differentiated into macrophages (with 2.5 nM PMA treatment) were cultivated in RPMI medium (Control group), submitted to tolerance (500 ng/ml of LPS 24 h before challenge with 1000 ng/ml of LPS * Tolerant group) and challenge (1000 ng/ml of LPS * D group) during 24 h. NO production was inhibited by addition of 100 μM of LNAME. The HAT activity and cytokine production (IL-6) were measured with biochemistry kits. Histone acetylated H3 and H4 were analyzed by western blotting.
Results: Tolerance reduced HAT activity compared with group directly challenged ($P < 0.05$). The H4 acetylated was maintained at basal levels in the tolerant group and increased in the D group ($P < 0.05$). However, the tolerance increases the acetylation of Histone H3 in a NO-dependent response. Similarly, the IL-6 release was reduced by induction of tolerance ($P < 0.05$ versus D). However, this effect was abolished by inhibition of NO production.
Conclusions: The induction of tolerance reduces HAT activity and cytokine production. The tolerance triggers a complex epigenetic modulation dependent of Nitric Oxide.

P1291

Epigenetic regulation of cytokine production in endotoxin tolerance and sepsis

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Purpose/Objective: One mechanism for host protection is endotoxin tolerance (ET), defined by a transient unresponsiveness to endotoxin (LPS) following a first exposure to this stimulus. While ET seems to be a protective mechanism against excessive inflammation, it plays a major role in death of sepsis patients suffering from secondary infections. In clinics, a reactivation of the immune system is necessary to overcome ET.

A recent publication suggested epigenetic modifications on histones which determine gene expression in endotoxin-tolerant murine macrophages. The aim of our study is to understand ET on the chromatin level in human monocytes and how the production of specific cytokines (e.g. IL6 and TNFα) is switched off during tolerance induction. This would give new insights in the reactivation of the immune system leading to new biomarkers or treatment strategies of sepsis patients.

Materials and methods: For induction of ET, human $CD14^+$ monocytes were desensitized with a preliminary treatment of LPS overnight followed by a second LPS challenge on day two. Subsequently, chromatin remodeling involving histone modifications was analyzed by chromatin-immunoprecipitation (ChIP).

Results: In contrast to murine macrophages, our results did not reveal differences between endotoxin responsive and endotoxin tolerant human monocytes in the methylation of histone 3 at lysine 4 (H3K4me3) – a marker for open chromatin. It was still present in the promoter regions of IL-6 and TNFα in desensitized cells. ChIP-Sequencing using an antibody directed to H3K4me3 confirmed that there are no differences in the promotor region of IL-6 and TNFα between the control group (unstimulated) and the LPS-treated group in terms of this particular modification. However, the promotor region of interleukin-1 receptor antagonist (IL1RN) and IL-1β showed an enrichment of H3K4me3 in the LPS-treated group indicating a positive feedback in transcription.

Conclusions: These results suggest that histone modifications (here H3K4me3) seem to play a role in induction of cytokine production in at least a subset of cytokines also in human monocytes. However, a combination of different ChIP assays combining positive and negative histone markers and key transcription factors (e.g. NF-κB) is necessary to understand the complex picture of induction and/or inhibition of cytokine production in ET.

P1293

Immune suppression in sepsis: is there a role of regulatory T cells?

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Purpose/Objective: Despite progress in intensive care therapy, sepsis remains the third common cause of death in Germany. While the hyperinflammatory first phase of sepsis (SIRS) can be controlled by supportive therapies, many patients die in the subsequent hypoinflammatory phase (CARS). We are interested in CARS as it is less well described but characterized by high susceptibility to secondary infections. It is well documented that in sepsis many lymphocytes undergo apoptosis contributing to the hypoinflammatory milieu. The innate immune response is impaired as demonstrated in mice by a reduced capacity of TNF production after sepsis. Moreover, the fraction of regulatory T cells (Tregs) is increased and the cells are activated early after sepsis induction.

Materials and methods: To investigate CARS, we have immunized animals after sepsis induction (AP, acute peritonitis, CASP, colon ascendens stent peritonitis) and analyzed the antigen-specific humoral immune response. To investigate if regulatory T cells play a role in the observed immune suppression we depleted Tregs after sepsis induction and before antigen-specific priming.

Results: The antigen-specific humoral immune response was strongly impaired in septic mice, indicated by a decrease of specific antibody titres. Additionally, the affinity maturation of the produced antigen-specific IgG antibodies was reduced and the migration of specific plasma cells into the bone marrow was impaired in these mice. Moreover, also T cells were affected, indicated by a reduced antigen-specific recall response *in vitro*.

Retrospective comparison of immune suppressed and immune competent animals showed not only a more severe course of disease but also an increase of total IgG concentration in immune suppressed animals. Moreover, splenic Tregs were more activated in these mice.

Treg-depletion before priming partially restored antigen-specific humoral immune response and increased affinity maturation. Additionally, the antigen-specific T cell recall response was also re-established.

Conclusions: The results indicate a role of Tregs in immunoparalysis after sepsis. However, other regulatory mechanisms appear to be involved as the antigen-specific humoral and T cell response was not restored completely.

P1294

LPS internalization mediated by Syk signals in lung epithelial cells

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Purpose/Objective: Toll-like receptor 4 (TLR4) was identified as a receptor for lipopolysaccharide (LPS) because mouse strains with nonfunctional or deficient TLR4 are endotoxin hyporesponsive. However, clinical trials using either anti-LPS antibodies or TLR4 inhibitors failed to save patients with LPS-induced sepsis. Mechanisms other than TLR4 may be responsible for the sustained inflammatory responses induced by LPS.

Materials and methods: Human lung epithelial cells were cultured on glass chamber slides overnight, incubated with the spleen tyrosine kinase (Syk) inhibitor BAY 61-3606 for 30 min, and then stimulated with BODIPY-LPS for 4 h. Cells were fixed for F-actin staining with Rhodamin-Phalloidin. Fluorescen images were taken under a confocal microscope and analyzed by the microscopy software ZEN lite 2011. IL-8 production was measured in culture supernatant from additional experiments without cell fixation. C57BL/10ScSnJ mice (Tlr4 wild type) were anesthetized with sevofluorane and divided into three groups. (1) LPS intratracheally administered concurrently with BAY 61-3606, with a second dose of BAY 61-3606 administered 2 h later. (2) LPS intratracheal instillation administered concurrently with saline I.P. injection, followed by a second I.P. administration of saline 2 h later. (3) BAY 61-3606 administered alone at time 0 and 2 h. Mice were sacrificed 4 h after LPS instillation. Immunohistochemistry of lung histology was analyzed for LPS internalization (goat anti-LPS antibody) and colocalization with the epithelial cell marker cytokeratin 8 (mAb), followed by Alexa-conjugated anti-goat IgG and Cy3 anti-mouse IgG). The nuclei were counterstained with DAPI.

Results: We demonstrate that human lung epithelial cells do not express surface but intracellular TLR4 and these cells still produce cytokines in response to stimulation with LPS. LPS was internalized

and co-localized with F-actin by human lung epithelial cells, leading to sustained IL-8 production. The LPS internalization was attenuated by the administration of BAY 61-3606. *In vivo* conditions, the LPS internalization and cytokine production were largely attenuated in Syk deficient mice. Furthermore, the LPS-induced cytokine response is reduced by the pharmacological intervention with BAY 61-3606 *in vivo* mouse model of endotoxemia.
Conclusions: These results suggest that Syk may act as a key molecule for LPS internalization that mediates subsequent inflammatory responses through TLR4-independent mechanisms.

P1295
LPS/endotoxemia leads to an altered activation threshold and signal transduction in splenic T-cells

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Purpose/Objective: Systemic inflammation or sepsis is a fatal condition that often literally overruns the patients« natural defense system. Following an initial burst of the innate immune system, the patients« adaptive immune system enters a protracted stage of paralysis. It is generally assumed that T-cells are compromised in their ability to fight infections in later stages of sepsis but if and how T-cell function affects sepsis outcome is still disputed. We have characterized T-cell paralysis/behaviour in LPS induced endotoxemia in an attempt to decipher the molecular mechanisms underlying this process and the role of T-cell paralysis in disease progression.
Materials and methods: Sublethal LPS doses were injected in CL57/B6 mice. Animals were continuously monitored and sacrificed 9 days after insult. CD4/CD8 positive T-cells were isolated from the spleen via automated magnetic separation and extensively characterized by FACS (phenotype, proliferation). To discern subtle changes in the sensitivity of T-cell signalling the isolated T-cells were challenged with a range of TCR-agonists including stimuli that are physiologically inert owing either to too low or too high TCR activation potency as well as physiologically active stimuli with intermediated TCR stimulation strength.
Results: Total splenic CD4/CD8 T-cell numbers were unaltered in LPS-treated mice, as were Treg ($CD4^+$, $CD25^+$, $Foxp3^+$) numbers, but there was a slight reduction of the CD4/CD8 ratio. As expected, the pattern of activation marker induction (CD25, CD69, CD137) and proliferation following *ex vivo* stimulation with the various agonists reflected the gradual increase in TCR stimulation strength of the individual cues. Importantly, only stimuli with intermediate, 'physiological' affinity for the TCR lead to expansion and proliferation of T-cells from mock-treated animals. Notably, T-cells from the LPS treated mice proliferated in response to unphysiologically 'weak' agonists and showed no signs of paralysis. This remarkable behaviour is accompanied by unconventional signalling downstream of the TCR which can, in part, explain the altered characteristics of the T-cells.
Conclusions: We propose that LPS/endotoxemia primes T-lymphocytes leading to a lower signal threshold for TCR stimulation and T-cell activation. Recapitulation of the same experimental program in *bona fide* animal models for sepsis/peritonitis is currently underway.

P1296
MyD88 is crucial for inflammation during obesity and for severity of sepsis

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Purpose/Objective: Obesity is a complex condition, affecting individuals of all ages and is characterized by a moderate state of chronic inflammation, with increased levels of several pro-inflammatory cytokines and acute phase proteins that maintain this inflammatory state. Obesity has also been shown to be a risk factor 'dose dependent' for morbidity and mortality in sepsis; although, little is known about the specific role of obesity in innate immunity activation, cellular and tissue dysfunction that are observed in sepsis. Our goal is to study the relationship between obesity and the regulation of immune response in sepsis in order to contribute for the understanding of the mechanisms involved in immune regulation of the inflammatory response of sepsis in obese individuals.
Materials and methods: We used a model of obesity induced by high fat diet in C57BL/6 and MyD88 knockout (KO) mice 60 days, and then they were submitted to sepsis by caecal ligation and puncture (CLP) with two perforations using 23G needles and sacrificed 24 h after sepsis induction.
Results: MyD88 KO mice showed higher weight gain and increased adipocyte size and reduced inflammation as compared to wild type mice. After sepsis, these mice were protected with longer survival, decreased of systemic inflammation, less macrophage infiltration in white adipose tissue and decreased gene and protein expression of pro-inflammatory molecules IL-1β, TNF-α, IL-6 and KC. In addition, we observed a significant increase in gene expression of arginase-1 and decreased of MCP1. The inflammation in these mice after sepsis appears to be reduced by a significant increase in gene expression of PPAR-γ, Glut4, LPL and FABP4, possibly by inhibiting NF-κB pathway.
Conclusions: Here, we concluded that the innate immunity mainly through MyD88 that plays an important role in obesity and inflammatory status in adipose tissue and after a severe inflammatory stimulus.
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P1297
Obesity alters sepsis induced pulmonary inflammation

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Purpose/Objective: Sepsis is a severe disease that represents a significant healthcare burden worldwide, while obesity has reached epidemic proportions over the last few decades. Although the mechanism is uncharted, it is known that obesity increases morbidity and mortality in sepsis through its multiple effects on many organ systems, including pulmonary function. Our aim was to investigate the effects of obesity in systemic and pulmonary inflammatory process in an experimental model of endotoxemic shock.
Materials and methods: Animals were fed a high fat diet (30% of fat) for 6 weeks and then injected with 15 mg/kg LPS i.p. They were euthanatized after 6, 24 and 48 h. Inflammation was characterized by measurement of plasma and pulmonary cytokines. The mRNA

expression of cytokines and tissue remodeling proteins was determined by real time PCR.
Results: Obesity decreased the survival rate of the animals 24 h after LPS injection. There was higher plasma concentration of IL1-beta, IL-6 and TNF-alpha in these animals. Furthermore, there was higher concentration of IL-6 in the obese mice's lungs after 6 h of endotoxemia. However, the mRNA expression of pro-inflammatory factors (IL-6, TNF-alpha, IL-1beta and MMP9) was lower, suggesting they may be converted to proteins. Obese mice presented higher mRNA expression of TGF-beta after 6 h, indicating a reparative process.
Conclusions: Obesity may be an additional complication factor in sepsis induced pulmonary inflammation.

P1299
Sepsis increases autoantibody titers in mice – antibody repertoire analysis at the single cell level

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Purpose/Objective: Sepsis is associated with immunosuppression due to lymphocyte apoptosis. Especially $CD4^+$ T cell-, B cell- and dendritic cell numbers decrease dramatically. Interestingly, in our polymicrobial sepsis model (Colon ascendens stent peritonitis, CASP) there is a strong increase in serum IgM and, a few days later, of IgG concentrations that reach maximal values 14 days after sepsis induction. Here, we investigated the specificity of the antibody response.
Materials and methods: For this, sera of septic mice were tested for IgM and IgG bindingto bacterial, foreign and self-antigens. Futhermore, splenocytes of septic mice were fused with myeloma cells to generate monoclonal B cell hybridomas. The resulting monoclonal antibodies were tested for binding to HEp-2 cells by immunofluorescence microscopy (a diagnostic tool for autoantibody screening) and for binding to a panel of bacterial, foreign and self-antigens (ELISA). In addition, immunoglobulin (Ig)-genes were sequenced to determine (1) whether the hybridomas were really monoclonal, (2) the usage of Ig-gene segments and (3) the frequency of somatic hypermutations.
Results: In serum we found autoreactive antibodies as well as antibodies directed against antigens (e.g. TNP, OVA) that mice had never been in contact with. Because it is impossible to discriminate between a polyclonal response and polyreactive antibodies in serum, monoclonal hybridomas of splenic B-cells were generated. These cells produced a large variety of autoreactive IgM and fewer autoreactive IgG. ELISA showed polyreactive IgM for the tested antigens. No antibodies with a unique specificity for any of the tested bacterial antigens were found. Sequencing revealed germ line or near germ line Ig-sequences for IgM and IgG.
Conclusions: We conclude, that sepsis activates B cells, which mostly produce polyreactive IgM binding to bacterial, self and foreign antigens. These data raise the question to which degree the humoral immune response in peritoneal sepsis is driven by specific antigen recognition (BCR) or by polyclonal stimuli (PRR), and which B cell subsets get activated.

P1302
Vav1 guards against excessive acute macrophage immune response in experimental septic shock and peritonitis

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Purpose/Objective: Overwhelming immune responses mediated by activated macrophages (MΦ) play a key role in acute inflammations and septic shock. Vav1, a family member of RhoGEFs, is mainly expressed in hematopoetic cells and exhibit scaffold protein function, GEF activity and participates in transcriptional active complexes. Furthermore, compared to the well characterized function of Vav1 in T cells, Vav1 mediates migration and cytoskeleton reorganization of MΦ. However, the capacity of Vav1 to influence early MΦ activity in the context of sepsis and acute inflammation has not been investigated extensively.
Materials and methods: In order to characterize Vav1 in MΦ, we analyzed $Vav1^{-/-}$ mice in LPS induced septic shock. ELISA, ChIP, Co-Immunoprecipitation and FACS analysis were used to specify behavior of *in vivo* or *in vitro* LPS stimulated peritoneal $CD11b^+$ $Vav1^{-/-}$ MΦ.
Results: Unexpectedly, in LPS induced septic shock Vav1 deficiency resulted in increased susceptibility and $Vav1^{-/-}$ mice could be characterized by increased systemic levels of IL-6. FACS analysis of whole blood and peritoneal lavage confirmed MΦ as source of IL-6. In accordance with our *in vivo* data, *in vitro* LPS stimulated peritoneal $CD11b^+$ $Vav1^{-/-}$ MΦ secreted significantly more IL-6 than WT MΦ, while in the same setting TNF-α secretion was not markedly affected. LPS/Actinomycin D treatment of WT- and $Vav1^{-/-}$ MΦ emphasized that Vav1 modulated IL-6 release on a transcriptional level. Our data excluded classical IL-6 regulation factors, like NFκB, as source of enhanced IL-6 secretion in $Vav1^{-/-}$ MΦ. Interestingly, Vav1 was located in the cytoplasm as well as in nuclei of MΦ and showed an association with HSF1 and the IL-6 promoter. Using a mutated IL-6 promoter construct, we were able to stress the functional relevance of Vav1/HSF1 association and DNA interaction for regulation of IL-6 expression.
Conclusions: Vav1 emerges as a crucial regulator protein of the early state of peritonitis and thus sepsis via dampening LPS induced IL-6 expression in MΦ. Strengthening of Vav1 signalling in the innate immune system might therefore represent an effective tool to regulate overwhelming IL-6 secretion in the context of septic peritonitis.

Poster Session: Tuberculosis

P1303

A 24 h-HBHA-specific interferon-gamma release assay for the detection of latent tuberculosis

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Purpose/Objective: Treatment of latent tuberculosis infection (LTBI) in target populations is one of the current WHO strategies to prevent active tuberculosis (TB) and reduce the *Mycobacterium tuberculosis (Mtb)* reservoir. Powerful LTBI screening tools are therefore indispensable. An interferon-gamma release assay (IGRA) in response to 4 day stimulation by the latency antigen Heparin-binding haemagglutinin (HBHA) has proven its potential. Adapting this assay for routine laboratory testing is essential. We evaluated its possible optimization through reduction of incubation time and the addition of co-stimulatory cytokine IL-7. These upgrades were analyzed in terms of immunological response and efficacy.
Materials and methods: A total of 95 patients were recruited from three Brussels based university hospitals, and divided into LTBI patients (defined according to CDC tuberculin-skin test guidelines), TB patients, healthy controls and patients with undetermined *Mtb* infection status. HBHA-specific IGRAs, with the classical 4 day stimulation and a 24 h stimulation with concomitant IL-7, were performed simultaneously on peripheral blood mononuclear cells isolated from participants' blood samples.
Results: Optimal IL-7 dosage, determined by dose-response curves, was 1 ng/ml. Correlation between the classical 4 day assay and the 24 h/IL-7 assay was high. Phenotypes of IFN-γ producing cells are currently being investigated through flow cytometry.
Conclusions: A 24 h/IL-7 interferon gamma release assay in response to HBHA is a promising candidate for the detection of latent tuberculosis. Greater numbers of LTBI and TB patients are presently being recruited to achieve statistically significant numbers and establish discriminatory IFN-γ concentration cut-offs between LTBI, TB and healthy controls.

P1304

A role for macrophage arginase-1 expression in immunopathology in tuberculosis granulomas

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Purpose/Objective: Granulomas in the lung are organized aggregates of immune cells and the hallmark of human tuberculosis (TB). These aggregates contain mainly non-infected and *Mycobacterium tuberculosis (Mtb)*-infected macrophages as well as *Mtb*-specific T cells. *Mtb* can persist within macrophages in granulomas due to insufficiencies of macrophage anti-microbial mechanisms. One such mechanism, the production of nitric oxide (NO) by nitric oxide synthase-2 (NOS2) via L-arginine and oxygen (O_2) is thought to be suboptimal in hypoxic regions in granulomas. Arginase-1 (Arg-1) also metabolizes L-arginine but does not require O_2 as a substrate and can thus function in hypoxic conditions. Arg-1 can act as a suppressor of acquired immune responses by depriving proximal T cells of L-arginine. We investigated the role of Arg-1 in tuberculosis pathogenesis using a murine model of granuloma formation.
Materials and methods: $NOS2^{-/-}$ C57BL6 mice, but not WT mice, develop lung granulomas similar to humans on *Mtb*- dermal infection and temporary blocking of IFN-γ. Using this model, we evaluated the role of Arg-1 in immunopathology in TB granulomas by flow cytometry analysis, histopathology, western blot, HLPC, enzymatic activity assay, measurement of local cytokine and chemokine production and quantification of bacterial burden.
Results: We found Arg-1-expressing cells in potentially hypoxic regions of lung TB granulomas in humans and non-human primates. Macrophages expressing Arg-1 were analogously present in human like-hypoxic TB granulomas in the lungs of $NOS2^{-/-}$ mice. Expression and activity of Arg-1 in this model correlated with decreased L-arginine and increased L-ornithine, spermidine and putrescine levels in the lung. Decreased expression of Ki-67 by T cells in hypoxic regions of granulomas indicated inhibition of T cell activation and proliferation in these locations. Furthermore, reduced IL-2 and elevated IL-10 production by lung cells in response to ESAT-6 stimulation support a micro-environment of T cell suppression.
Conclusions: These and preliminary data suggests that macrophages expressing Arg-1 modulate immunopathology and tissue destruction in TB granulomas by dampening T cell activity in hypoxic regions.

P1305

A two-compartment system allows lymphatic tissues to control *M. tuberculosis* infection in the peripheral organs

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Purpose/Objective: There is a paradox in the low dose aerosol model of experimental murine model of TB. At week 3, the bacillary load (BL) growth is controlled in lungs, but still grows in spleen, where the immune response is thought to be triggered. A '2-compartment' hypothesis has been raised, in which lymphoid tissues infection shall take place in a compartment, 'previous' to where the specific lymphocytes are generated. This proliferation shall not protect their origin organs: for being released to the efferent vessels, the lymphocytes are attracted in those organs with a more intense inflammatory response, thus where the BL is higher. Only when the BL is controlled and the attraction capacity is reduced, the lymphocytes will come back to the infected compartment of the origin organs to control the BL.
Materials and methods: 6-weeks old female C57BL/6 mice were infected via aerosol (aer) with 20–50 bacilli using a Middlebrook device or ev (10^2, 10^4, 10^5 CFU/ml with *M. tuberculosis* H37Rv Pasteur strain. The animals were sacrificed at sequential timepoints: aer experiment (exp) everyday from day 10 to 22 post infection (pi); ev exp at days 0 and 2, 5, 8, 11, 14, 17, 20, 28 and 42 pi. The BL and specific immune responses were measured in lungs, spleens and lymph nodes, by culturing them in 7H11 agar plates and ELISPOT assays, respectively. An 'in silico' model was designed considering the generation and life of lymphocytes, the immune response and BL in tissue, and the flows among lymph and blood vessels of the 3 organs.
Results: The use of the highest infective dose ev (10^5 CFU) demonstrated 5 days is the elapse time needed to trigger the proliferation of effector lymphocytes in spleen, while the other experiments suggested the presence of a BL threshold around 10^3 CFUs in tissue needed to be overcome. The experiments confirmed that even when the lymphatic tissues are able to generate specific lymphocytes, the BL can still grow

there at the same time. Integration of this data in a mathematical model explains this paradox after considering the dynamics of fluxes among the three organs.

Conclusions: The results confirm the original hypothesis of the two compartment system in lymphatic tissues, which prioritizes the control of the infection in lungs and avoids the self-resolution of their own infection processes which would leave non-lymphatic organs defenseless.

P1306

Apoptosis and TNF receptor expression in alveolar macrophages during *Mycobacterium bovis* infection in mice

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Purpose/Objective: Apoptosis of infected macrophages has been reported as an effective host strategy to control the growth of intracellular pathogens, including pathogenic mycobacteria. TNF-α may play an important role in the modulation of apoptosis of infected macrophages. TNF-α exerts its biological activities via two distinct cell surface receptors, the 55-kDa receptor (TNF-R1) and the 75-kDa receptor (TNF-R2) whose extracellular domain can be released by proteolysis forming soluble TNF receptors (sTNFR-1 and sTNFR-2). The signaling through TNF-R1 initiates the majority of the biological functions of TNF-α, leading to either cell death or survival whereas TNF-R2 mediates primarily survival signals. In the present work, the expression of TNF-α receptors and the apoptosis of alveolar macrophages were investigated during infection with virulent and avirulent mycobacteria in mice.

Materials and methods: C57BL/6 mice were intratracheally infected with avirulent (BCG Moreau) and virulent (ATCC19274) strains of *M. bovis*. Mice were killed 3 and 7 days after infection and lung cells obtained by *Bronchoalveolar* lavage (BAL). Alveolar macrophages were differentiated according to their CD11b/CD11c profiles and apoptosis was assessed by flow cytometry using Annexin V-FITC/PI labeling. Cell surface TNFR1 and TNFR2 were studied using FITC-conjugated anti-mouse CD120a (TNFR1) and PE-conjugated anti-mouse CD120b (TNFR2). Levels of soluble TNF receptors (sTNF-R1 and sTNFR2) in BAL were measured by ELISA.

Results: A significant increase of apoptosis and high expression of TNFR1 were observed in alveolar macrophages at 3 and 7 days after infection with *M. bovis* BCG but only on day 7 in mice infected with the virulent *M. bovis*. Low surface expression of TNFR1 and increased levels of sTNFR1 on day 3 after infection by the virulent strain were associated with reduced rates of apoptotic macrophages. TNFR2 expression was lower on days 3 and 7, which was accompanied by increased levels of sTNFR2 in mice infected with both strains of *M. bovis*.

Conclusions: These results suggest a potential role for TNFR1 in mycobacteria induced alveolar macrophage apoptosis *in vivo* in a strain dependent manner.

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P1307

BCG vaccination inducing long-term protection against *Mycobacterium tuberculosis* correlates with the differentiation of IL-17/IL-2/TNF producing T cells

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Purpose/Objective: Tuberculosis is responsible for approximately 2 million deaths each year. *Mycobacterium bovis* BCG is the only available vaccine to prevent Tuberculosis. The re-emergence of the disease, as well as the appearance of high rates of multidrug resistant strains, makes the development of novel vaccines imperative.

It is documented that the differentiation of polyfunctional Th1 cells, namely those producing IFN-g/IL-2/TNF, or IL-2/TNF, are of utmost importance in the control of *M. tuberculosis* infection. However, recently a new T cell population, producing IL-17 (Th17) has also been reported to play a role in both primary and secondary immune responses to *M. tuberculosis* infection.

Materials and methods: To dissect further the role of Th17 cells in vaccination, C57BL/6 mice were vaccinated with BCG for 21 or 120 days prior to *M. tuberculosis* intranasal infection. At different days post-infection, the number of ESAT-6 and Ag85-specific T cells producing IFN-g or IL-17 was measured by ELISPOT and the frequency of IFN-g, IL-2, TNF and IL-17 producing T cells was analysed by flow cytometry.

Results: Our results show that protection was associated with a reduction in the number of both Th1 and Th17 cells specific for Ag85 or ESAT-6, as compared to non-vaccinated mice. Moreover, the differentiation of Ag85 specific effector/memory $CD4^+$ T cells, induced by BCG vaccination, influenced the primary response to ESAT-6, from *M. tuberculosis*.

In addition, our results show that although the frequency of the IFN-g/IL-2/TNF producing T cells was increased early after infection in both vaccinated and unvaccinated mice, a sustained increase in the frequency of polyfunctional T cells producing IL-17/IL-2/TNF was only seen in mice vaccinated with BCG 120 days prior to infection.

Conclusions: These data suggest that the period between vaccination and challenge can impact the longevity and type of the polyfunctional response, with influence in the level of protection.

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P1308

Correlates of immune protection in *Mycobacterium tuberculosis*

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Purpose/Objective: Current T cell based interferon-gamma release assays (IGRA) to detect TB antigen specific responses cannot distinguish between active and latent TB infection. In addition, more robust biomarkers of active TB are needed in immune-compromised populations such as those with HIV/TB co-infection. Our lab has recently shown that Th1Th17 CD4 T cells are significantly reduced in HIV-1 infected patients. Also, IFN-γ and IL-2 PPD responses are significantly reduced in ART naïve patients but are partially restored with successful ART. Therefore, we sought to determine whether the loss of Th1Th17

CD4 T cells and reduced cytokine responses were also found inpatients with TB infection, with the aim of identifying potential biomarkers to distinguish active and latent TB.

Materials and methods: Study participants comprised of healthy controls ($n = 12$), active tuberculsosis (ATB) ($n = 10$), latent tuberculosis (LTB) ($n = 10$), HIV-1 patients with ATB (HIV/ATB) ($n = 10$), and LTB (HIV/LTB) ($n = 7$). Whole venous blood was used to look at CD4 T cell subsets (Th1, Th2, Th17, Th1Th17, and memory) via flow cytometry. ELISPOT (Gen-probe) was used to measure IFN-g and IL-2 cytokine responses to PPD, ESTAT-6 and CFP-10.

Results: We found no significant differences in the proportion of TH1, Th2, TH17 or Th1Th17 cells between patients with ATB and LTB for both HIV-1 positive and negative individuals. CD4 T cell expression of CXCR3 was increased in HIV/ATB patients compared to HIV/LTB (media $n = 46.2\%$ versus 35.0%) ($P < 0.05$).

ELISPOT analysis showed that there were no differences in IFN-g or IL-2response to ESTAT-6, CFP-10 or PPD for ATB/HIV patients compared to HIV/LTB. In contrast, among HIV negative individuals, LTB patients had significantly increased IFN-g (media $n = 388$ versus 50 SFC/10^6 PBMCs) responses to ESTAT-6 compared with ATB patients ($P < 0.01$). Patients with LTB had consistently higher cytokine responses compared to HIV/LTB co-infected patients. Median IFN-g responses to ESTAT-6 were 388 versus 13 and CFP-10 were 930 versus 16 SFC/10^6 PBMCs ($P < 0.01$). Median IL-2 responses to ESTAT-6 were 291 versus 18 andCFP-10 were 304 versus 4 SFC/10^6 PBMCs ($P < 0.01$).

Conclusions: Preliminary data suggest that ESTAT-6 ELISPOT counts of IFN-g responses may enablethe distinction between active and latent TB in HIV-1 negative individuals.

P1310

M2 macrophages and LTB_4 are factors associated with the lower lung *Mycobacterium tuberculosis* counts in the infected and allergic mice

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Purpose/Objective: Epidemiological data show that the incidence of allergic response is lower in Tuberculosis Skin Test positive individuals. However, Th2 immune response, characteristic of allergy, may down modulate the protection mediated by Th1 cellular immune response in tuberculosis (TB).

Materials and methods: In attempt to study how it would be the outcome of the allergic response in the *Mycobacterium tuberculosis* infection, we sensitized and challenged BALB/c mice with Ovalbumin (OVA) after 30 days of intra-tracheal *M. tuberculosis* infection.

Results: Infected, allergic mice (TB/OVA group) presented bacterial load significantly lower than infected mice (TB group) 54 and 80 days post-infection. Because the decrease in the bacterial load was not dependent on IFN-gamma and IL-17 secretion or alum adjuvant, we hypothesized that this phenomenon was dependent on leukotrienes production, that play a role in TB and asthma. There was a significant higher secretion of LTB_4 between TB/OVA and TB groups. Treatment with MK-886 compound, which inhibits the 5-Lipoxygenase pathway, abolished the reduction of CFU counts, previously observed when compared to respective non-treated groups. A histological analysis showed a reduction in cell influx in the lung granuloma areas of TB/OVA mice. Because foamy macrophages are predominant leukocytes in the TB granuloma, we evaluated populations of M1 and M2 macrophages. There was a significant increase in the transcription of genes associated with the M2 phenotype in the lungs of TB/OVA group compared to TB group.

Conclusions: Therefore, the reduction in the lung CFU counts of infected and allergen sensitized mice appears to be dependent on LTB_4 and M2 macrophage. Further experiments are undergoing to depict the biological meaning of these findings.

P1311

Macrophage bioenergetics is differentially regulated by *M. tuberculosis* virulence factors

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Purpose/Objective: To analyse the bioenergetic consequences of macrophage stimulation with different *M. tuberculosis* proteins, regarded as posible virulence factors.

Materials and methods: J774 cells were stimulated with *M. tuberculosis* recombinant proteins p27, PE_PGRS1, PE_PGR33, and MT_1866 or left untreated. Nitric Oxide (NO) and ATP synthesis, glycolysis (lactate production), mitochondrial respiration, and mitochondrial dynamics were evaluated at different time points post-stimulation.

Results: p27 and PE_PGRS1 induced NO synthesis, diminished mitochondrial respiration and enhanced glycolysis. In contrast, PE_PGRS33 failed to induce NO production and glycolysis but increased mitochondrial respiration. MT_1866 induced NO production and glycolysis only slightly. PE_PGRS33 induced hyperfusion of mitochondrial networks by 4 h post-stimulation, whereas P27 induced mitochondrial fragmentation. These mitochondrial dynamics correlated with the rate of oxygen consumption.

Conclusions: Results suggest that different virulence factors from the same pathogen (*M. tuberculosis*) have different and seemingly opposing effects on the bioenergetics of the host cell; the interplay of these actions could ultimately determine the outcome of host-pathogen interactions.

P1312

MMP-10 is increased in respiratory secretions in pulmonary TB and is specifically upregulated by pathogenic Mtb in human macrophages

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Purpose/Objective: Morbidity in *Mycobacterium tuberculosis* (*Mtb*) infection results from pulmonary matrix destruction. Matrix metalloproteinases (MMPs) degrade all lung extracellular matrix components and are implicated in tissue destruction in tuberculosis (TB). MMP-10 is a stromelysin which can activate the collagenases, MMPs -1, -8, -13. Therefore we hypothesise that MMP-10 has a critical role in immunopathology in human tuberculosis. We investigated MMP-10 concentrations in patients with pulmonary TB and MMP-10 regulation in a cellular model of *Mtb* infection.

Materials and methods: MMP-10 in induced sputum and bronchoalveolar lavage fluid (BALF) of patients with TB ($n = 38$) and controls ($n = 35$) was measured by Luminex bead array. Human macrophages (MDMs) were infected with live virulent H37Rv *Mtb* or attenuated vaccine Bacillus Calmette-Gu*rin (BCG). p38/ERK MAP kinase and Pi3 kinase signalling pathways were inhibited chemically with SB203580, PD98059 and LY294002 respectively. MMP-10 and TNF alpha secretion was measured by ELISA and MMP-10 mRNA by RT-PCR.

Results: The median level of MMP-10 in sputum/BALF of TB patients was 5173 pcg/ml (interquartile range 883−13286 pcg/ml) compared to

2217 pcg/ml (interquartile range 528–4940 pcg/ml) in controls ($P = 0.01$). *Mtb* upregulates MMP-10 gene expression and secretion from MDMs 14-fold relative to uninfected cells ($P < 0.005$) in a dose and time dependent manner. Inhibition of the p38 and ERK MAPK signalling pathways suppresses *Mtb* driven MMP-10 secretion in a dose dependent manner ($P < 0.005$ for each inhibitor), due to suppressed mRNA synthesis. Inhibition of the Pi3 kinase pathway has no effect on *Mtb* driven MMP-10 secretion. *Mtb* driven upregulation of MMP-10 secretion from MDMs is 3.5-fold greater than that by BCG ($P < 0.005$) and is secondary to reduced mRNA accumulation. In contrast, TNF alpha secretion by *Mtb* and BCG infected cells is equal.

Conclusions: MMP-10 concentration is increased in the respiratory secretions of patients with active pulmonary TB. Live virulent *Mtb* but not attenuated vaccine strain BCG specifically upregulates MMP-10, although both upregulate TNF alpha secretion equally. *Mtb* driven MMP-10 secretion is under the control of the p38 and ERK MAP kinase pathways. To our knowledge, these are the first data implicating MMP-10 in tissue destruction in pulmonary TB.

P1313

Mycobacterium tuberculosis strains are differentially recognized by TLRs with an impact on innate immune responses and the outcome of infection

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Purpose/Objective: Tuberculosis (TB) is characterized by a wide spectrum of disease outcomes. The Beijing (Bj) family of *Mycobacterium tuberculosis* (Mtb) strains is suggested to be more virulent than other Mtb genotypes and prone to elicit non-protective immune responses. Highly heterogeneous immune responses were reported upon infection of innate immune cells with Bj strains, but the molecular mechanisms underlying this heterogeneity are largely unknown. With this study, we aimed at investigating whether differential recognition of Mtb strains by toll-like receptors (TLRs) contributes to distinct innate immune responses with an impact on the outcome of infection.

Materials and methods: Bone marrow-derived macrophages generated from wild-type, TLR2 deficient or TLR4 deficient mice were infected with a panel of Mtb strains. At specific time-points post-infection the cytokine response was measured at the mRNA and protein levesl, by qPCR and ELISA respectively. Activation of specific signaling cascades was also assessed by Western Blot. Experimental infections were performed intra-nasally and the progression of infection measured by colony forming units counting, lung mRNA expression of specific genes and lung histology.

Results: We found Mtb strains that preferentially activate TLR2, and others that activate TLR4. This differential recognition is not unique to the Bj family, as we also found it within the Euro-American lineage of Mtb. Recognition of Mtb strains by TLR4 resulted in a distinct cytokine profile *in vitro* and *in vivo*, with specific production of Type I IFN. We also uncovered a novel protective role for TLR4 *in vivo* when infecting with TLR4 activating Bj Mtb strains.

Conclusions: Our findings provide the first molecular basis in host innate immune cells to, at least in part, explain the heterogeneity of responses to Mtb strains.

P1314

Mycobacterium tuberculosis-specific CD8 T cells are functionally and phenotypically different between latent infection and active disease

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Purpose/Objective: Tuberculosis (TB) is the second worldwide leading cause of death from an infectious disease after HIV infection. Protective immunity to *Mycobacterium tuberculosis* (*Mtb*) remains poorly understood and the role of *Mtb*-specific CD8 T-cells is controversial. We performed comprehensive functional and phenotypic characterizations of *Mtb*-specific CD8 T-cell responses in 273 subjects with either latent *Mtb* infection (LTBI) or active TB disease (TB) to assess their profile and relevance in TB.

Materials and methods: Using multi-parametric flow cytometry, we assessed *Mtb*-specific CD8 T-cell functional (production of IFN-gamma, IL-2 and TNF-alpha; proliferation capacity and cytotoxicity) and phenotypic (T-cell differentiation and exhaustion) profiles in cells isolated from peripheral blood and correlated these profiles with distinct clinical presentations.

Results: *Mtb*-specific CD8 T-cells were detected in most TB patients and few LTBI subjects (65% and 15%, respectively; $P < 0.00001$) and were of similar magnitude with a comparable cytokines profile (IFNg+TNFa+IL2-) in both groups. *Mtb*-specific CD8 T-cells were mostly T_{EMRA} (CD45RA$^+$ CCR7-) co-expressing 2B4 and CD160 in LTBI subjects and mostly T_{EM} (CD45RA-CCR7-) lacking PD-1/CD160/2B4 in TB patients. Furthermore, *Mtb*-specific CD8 T-cells mostly expressed very little perforin and granulysin but contained granzymes A and B or lacked all these cytotoxic markers in TB and LTBI subjects, respectively. However, *in vitro* expanded *Mtb*-specific CD8 T-cells acquired perforin, granulysin and granzymes. Finally, *Mtb*-specific CD8 T-cell responses were more robust and prone to proliferate in patients with extrapulmonary compared to pulmonary TB.

Conclusions: The clinical status and TB presentation are associated to specific profiles of *Mtb*-specific CD8 T-cell responses, thus indicating distinct dynamics between the mycobacteria, the CD8 T-cell response and the clinical outcome. Our data shed light on the controversial reached by studies performed in human and animal models, thus advancing the current knowledge on the complex dynamic of TB immunity.

P1315

Phagosome maturation induces the activation of NALP3 inflammasome which enhances the immunogenicity of BCG

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Purpose/Objective: Since *Mycobacterium bovis* Bacille Calmette-Gu*rin (BCG) is poorly efficient against TB, we have developed a mutant BCG with potentially stronger immunogenicity than wild-type BCG.

Materials and methods: Wild-type *M. bovis* BCG and deletion mutant deficient in the zinc-metalloprotease 1 (D*zmp1*) were grown in 7H9 broth. Bone-marrow dendritic cells (BMDCs) were cultured from femurs of mice as previously described. For *in-vitro* studies, BMDCs were infected with BCG of both strains overnight. In part, inflamma-

some inhibitors were added 30 min before addition of BCG to the BMDCs. For *in vivo* studies, 2×10^6 CFU for subcutaneous (s.c) immunisations and 10^5 CFU for intraperitoneal (i.p) infection were used. Twelve days (DM1) after s.c immunisations, mice were sacrificed and splenocytes restimulated. Analysis of restimulation was done by FACS and ELISA. Forty-eight hours after i.p infection, flow cytometric characterisation of cells within the peritoneal lavage was done.

Results: We found that the activation of the inflammasomes, was stronger in the *zmp1*-deficient strain. BCGD*zmp1* facilitated secretion of IL-1b from BMDCs was dependent on the NALP3 inflammasome. The inflammasome activation was dependent on cathepsin B while ROS played a minor role. Neutrophils have been suggested to affect specific T-cell responses against BCG and the recruitment of neutrophils is regulated by the inflammasome. In mice infected i.p. with BCG and BCGD*zmp1*, we indeed found that the *zmp1*-deficient strain had higher numbers of neutrophils in the peritoneal lavage. Moreover, animals that were s.c. immunised with BCGD*zmp1* had higher frequencies of IFNg-producing CD4 and CD8 T cells when compared to mice immunised with wild-type BCG. Also, re-stimulation of splenocytes *in vitro* showed increased antigen-specific IL-17 secretion in cells from mice immunised with BCG*zmp1*. When caspase-1-deficient mice were immunised, the increased Th1 and Th17 immune responses seen in wild-type mice were dampened further supporting that the presence of Zmp1 in BCG prevents efficient immune responses through inactivation of inflammasome.

Conclusions: By inhibiting inflammasome activation, BCG was unable to be an efficient vaccine against TB. We have clearly shown that with increased inflammasome activation we were able to induce better memory T cell response.

P1318

Relationship of IL-10 gene polymorphism (-1082A/G) with cytokine production and tuberculosis (TB) suceptibility in Polish population

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Purpose/Objective: Several genes coding for different cytokines may affect complex interaction between *M. tuberculosis* and host immune system. The aim of this study was to investigate the relationship of the single base change polymorphic variants identified in the promoter region of interleukin-10 gene (-1082 A/G) with cytokine production in whole blood cultures and TB susceptibility.

Materials and methods: We studied a Polish population of 191 volunteers including 32 patients with culture-proven pulmonary TB, 41 patients with non-mycobacterial lung diseases (non-TB), 71 healthy close TB contacts and 47 healthy control subjects. The QuantiFERON-TB Gold In Tube (QFT) assay was performed for all participants. Genotype frequencies for IL-10 (-1082G/A) polymorphism were analyzed using allele specific PCR method. The levels of IL-10 in whole blood cultures stimulated with mycobacterial antigens (PPD, ESAT-6, *M. bovis* BCG and *M. tuberculosis* $H_{37}R_v$ bacilli) were tested by ELISA.

Results: The frequency of latent *M. tuberculosis* infections was significantly increased among TB contacts (46%) as compared with non-TB patients (20%) and healthy volunteers unexposed to TB (13%). The distribution of the IL-10 genotypes (A/A, A/G and G/G) was similar in all groups being under the study. The 31% TB patients, 37% non-TB patients and 29% healthy controls were A/A homozygous. However, A/A homozygosity was found more frequently in QFT (+) than QFT(-) TB contacts (47% versus 10%, respectively).

The association between stimulated with mycobacterial antigens IL-10 levels and *IL-10* promoter polymorphism was noticed for neither healthy groups nor patients, however we observed significantly higher concentrations of the cytokine in cultures of blood from TB patients compared with healthy control subjects.

Conclusions: We conclude that polymorphic variants of *IL-10* gene may have possible impact on susceptibility to *M. tuberculosis* infection, but this polymorphism was not associated with progression of TB infection to disease. Mycobacteria driven IL-10 activity was not related to IL-10 gene variants. In contrast, an enhanced cytokine production in the group of TB patients was probably a consequence of disease manifestation.

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P1320

Risk stratification of latent tuberculosis defined by combined interferon gamma release assays

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Purpose/Objective: Most individuals infected with *Mycobacterium tuberculosis* develop latent tuberculosis infection (LTBI). Some may progress to active disease and would benefit from preventive treatment yet no means currently exists to predict who will reactivate. Here, we provide an approach to stratify LTBI based on IFN-g responses to two antigens, the Early-Secreted Antigen Target-6 (ESAT-6) and the latency antigen Heparin-Binding Haemagglutinin (HBHA).

Materials and methods: We retrospectively analyzed results from in-house IFN-g-release assays (IGRA) with HBHA and ESAT-6 performed during a 12-year period on serial blood samples (3−9) collected from 23 LTBI subjects in a low-TB incidence country. Both the kinetics of the absolute IFN-g concentrations secreted in response to each antigen and the dynamics of HBHA/ESAT-6-induced IFN-g concentrations ratios were examined.

Results: This analysis allowed the identification among the LTBI subjects of three major groups. Group A featured stable IGRA profiles with an HBHA/ESAT-6 ratio persistently higher than 1, and with high HBHA- and usually negative ESAT-6-IGRA responses throughout the study. Group B had changing HBHA/ESAT-6 ratios fluctuating from 0.0001 to 10 000, with both HBHA and ESAT-6 responses varying over time at least one time during the follow-up. Group C was characterized by a progressive disappearance of all responses.

Conclusions: By combining IGRAs with early and latency antigens, LTBI subjects can be stratified into different risk groups. We propose that disappearing responses indicate cure, that persistent responses to HBHA with HBHA/ESAT-6 ratios ≥1 represent stable LTBI subjects, whereas subjects with ratios varying from ≥1 to <1 should be closely monitored as they may represent the highest-risk group, as illustrated by a case report, and should therefore be prioritized for preventive treatment.

P1321

The degree of CD4 T cell differentiation as marker of tuberculosis severity and progression

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Purpose/Objective: For many infections, especially, chronic, evaluation of disease progression is an important objective. Antigenic and inflammatory stimuli released during infections induce T cell activation and differentiation from naïve to effector lymphocytes. Thus, it was hypothesized that the degree of T cell differentiation depends on infection severity (activity) and may be used for disease monitoring. In this study we addressed how the differentiation status of CD4 T cells changes during pulmonary tuberculosis (TB) and whether its evaluation may be used to monitor TB progression.
Materials and methods: The differentiation status of CD4 T cells was evaluated by analyzing the expression of CD45RO, CD62L, CD27, CD28, and CD57 markers on CD4 T cells. Based on the co-expression of these markers, naïve ($CD45RO^-$ $CD62L^+$), central memory (T_{CM}, $CD45RO^+CD62L^+$) and effector memory (T_{EM}, $CD45RO^+CD62L^-$) cells were identified; within the population of T_{EM} cells, early ($CD27^+$), late ($CD27^-$), senescent ($CD27^-$ $CD28^-$) and terminally differentiated ($CD27^-$ $CD57^+$) lymphocytes were detected.
Results: In TB patients a tendency towards a lower content of naïve CD4 T cells and an increase in the frequency of T_{EM} cells was registered. Within T_{EM} cells, different populations of cells predominated in patients with different TB severity. In patients with mild TB, early T_{EM} cells were increased; in patients with severe TB, late T_{EM} cells and their different subsets (i.e. senescent and terminally differentiated) accumulated ($P < 0.05$). Within a population of *Mtb*-specific cells, accumulation of late ($CD27^-$) T_{EM} cells was also observed in patients with more severe TB ($P < 0.0001$). Following effective TB treatment the frequencies of late T_{EM} decreased ($P < 0.01$).
Conclusions: Severe TB is associated with the accumulation of highly differentiated effector CD4 T cells. Evaluation of the degree of CD4 T cell differentiation represents a means to monitor TB progression and patient responsiveness to TB treatment.

P1322

TNFa/IL2 ratio discreminates latent from active tuberculosis in immunocompetent children

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Purpose/Objective: In clinical practice, distinguishing children with LTB from those with active early disease is of key importance in order to initiate an appropriate management of the patient. The aim of the study was to identify cytokine profiles that could differentiate LTB from TB disease.
Materials and methods: A Luminex assay was used to compare Th1 (IFNγ, TNFα, IL2) and Th2 (IL4, IL10, IL5, IL13) *in vitro* lymphocyte responses to *Mycobacterium tuberculosis* antigens (ESAT-6 CFP-10 and TB 7.7). Eighteen immunocompetent children were included. Six presented LTB (median age: 6.2 years) and 8 TB disease (median age: 5.7 years) following recent contact (≤3 months) with TB. Four age-matched children with recent contact but uninfected were also analysed as reference.
Results: Poor induction, by antigens, of IL4 and IL10 precluded analysis of these cytokines. IFNγ, TNFα, IL2, IL5 and IL13 were however clearly induced by antigens in 14/14 infected but in 0/4 uninfected children. Th1 cytokine levels were similar in LTB and TB disease (IFNγ: 12254 and 10495 pg/ml, IL2: 2097 and 1869 pg/ml, TNFα: 1020 and 2875 pg/ml, respectively). Th2 cytokine levels were similar or even higher in LTB (IL5:23 and 10 pg/ml, IL13: 284 and 109 pg/ml in LTB and TB disease, respectively). A positive correlation (spearman's rank correlation) between all cytokine levels, whatever Th1 or Th2, was observed in the 14 infected children. A higher (linear regression, $P < 0.01$) TNFα/IL2 ratio in TB disease discriminated 6/8 TB diseases from 6/6 LTB.
Conclusions: We do not confirm previous reports that suggested an association between progression and a biais toward Th2 responses. Instead, a higher TNFα response (involved in effector function) related to IL2 response (involved in protection) was found. The TNFα/IL2 ratio could help in staging *M. tuberculosis* infection following recent contact in immunocompetent children.

P1323

Vaccination with the Δmce2 mutant *M. bovis* strain induced in cattle protective immune response against *Mycobacterium bovis* challenge

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Purpose/Objective: Bovine tuberculosis (bTB) is not only serious animal and zoonotic disease that causes significant financial loss but also a public health hazard. Vaccination of cattle to control bTB is particularly demanding in high-prevalence zones where it is economically unfeasible to slaughter animals. Although previous studies with BCG in cattle have demonstrated reductions in disease severity to subsequent experimental challenge with virulent *M. bovis* strains, the protection induced by BCG was, in general, not complete. In this study we explore another attractive alternative that is to vaccinate cattle with a *M. bovis* mutant strain in the *mce2* operon of virulence. The protection efficacy of the candidate vaccine against *M. bovis* challenge as well the immune responses induced by the vaccine were tested in cattle and the results were compared to that obtained with BCG vaccination.
Materials and methods: Three groups of 5–6 Holstein-Fresian calves (3–4 months old) were inoculated subcutaneously in the side of the neck with either Δmce2, BCG Pasteur or vehicle. Eight weeks after vaccination, animals were infected with an *M. bovis* field strain by intratracheal instillation of 10^6 CFU as described previously. Sixteen weeks after infection, the calves were euthanized and then thin slices of lungs and lymph nodes of the head and pulmonary region were analyzed looking for granuloma formations. Macroscopic lesions were scored as previously described. At different points, post vaccination and prior to challenge, and post challenge, blood samples were taken for cytokines quantification, and T cell population analysis.
Results: The group of animals vaccinated with *M. bovis* Δmce2strain showed significantly reduced number of lesion as compared to the non vaccinated group. By contrary, the group vaccinated with BCG did not showed significantly differences to the non vaccinated group. By cross-sectional analysis of responses from those animals that presented lung lesions compared to animals that did not, we found that IL-17 was statistically higher expressed, before challenge, in blood of protected animals than in those non protected.
Conclusions: We found that *M. bovis* Δmce2strain better protected cattle against virulent *M. bovis* challenge than the vaccine strain BCG, in term of number and severity of lesions in lungs and lymph nodes.

Poster Session: Tumour Immunology

P1324

A dual role for the inflammatory chemokine CCL2/CCR2 pathway in controlling the infiltration of pro-tumor and anti-tumor leukocyte subsets

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Purpose/Objective: The identification of tumor-infiltrating leukocyte (TIL) subsets that either inhibit or promote tumor growth has been an important development in tumor immunology. It is thus critical to characterise the molecular cues that determine the balance between anti- versus pro-tumor leukocyte subsets within the tumor bed. In this study we aimed at identifying the chemokine signals that control leukocyte infiltration and the outcome of tumor development in experimental models.

Materials and methods: We transplanted the melanoma B16 cell line s.c. in wild-type and chemokine/chemokine receptor-deficient mice. We analysed B16 lesions for leukocyte infiltrates (including $CD8^+$, $CD4^+$ $Foxp3^-$ and $CD4^+$ $Foxp3^+$, and $\gamma\delta$ T cells; NK cells; macrophages and myeloid-derived suppressor cells, MDSCs) by FACS. Chemokines were profiled in B16 tumor extracts using a Quantibody Chemokine Array, and their functions were tested in chemotaxis assays *in vitro*.

Results: The chemokine CCL2 accumulated in B16 lesions from T cell-deficient mice, suggesting an unanticipated role in controlling T cell migration and tumor infiltration. Interestingly, this was specific to the $\gamma\delta$ T cell subset, which produced high levels of interferon-g and cytotoxic mediators (perforin and granzymes) within the tumor bed. Moreover, $\gamma\delta$ T cells expressed the counter-receptor CCR2 and migrated towards CCL2 *in vitro*, while CCL2 neutralization in B16 protein extracts completely inhibited $\gamma\delta$ T cell chemotaxis. To determine the functional relevance of CCL2/CCR2 *in vivo*, we established B16 tumors in CCR2-deficient versus WT mice. Analysis of TILs revealed significantly reduced numbers of $\gamma\delta$ T cells, macrophages and MDSCs in CCR2-deficient hosts. By contrast, the recruitment of $CD8^+$ or $CD4^+$ $Foxp3^+$ T cells was similar in both strains. To assess the impact of $\gamma\delta$ T cell infiltration on tumor development, we implanted B16 tumors in $\gamma\delta$ T cell-deficient mice, and observed increased (and more rapid) tumor growth.

Conclusions: Our data identified a novel anti-tumor role of CCL2/CCR2 based on the migration and infiltration of $\gamma\delta$ TILs endowed with potent interferon-γ production and cytotoxicity, which inhibited tumor growth *in vivo*. Moreover, since CCL2 also recruited pro-tumor leukocytes (macrophages and MDSCs), this work establishes a complex dual function of CCL2 in cancer immunity.

P1326

Activity of endoplasmic reticulum aminopeptidase 1 in malignant melanoma influences tumor recognition by autologous CD8+ T Cells

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Purpose/Objective: Endoplasmic reticulum aminopeptidase 1 (ERAP1) plays an important role in the generation of viral and parasitic $CD8^+$ T cell epitopes by trimming N-terminally elongated peptide precursors to the correct epitope length. In cervical carcinoma, low ERAP1 expression is associated with reduced overall survival, suggesting its involvement in tumor immune escape. However, the significance of ERAP1 in the generation of tumor antigen epitopes and anti-tumor immune responses remains still unclear.

The aim of the current study was to investigate if ERAP1 activity in melanoma cells influences autologous $CD8^+$ T cell activation.

Materials and methods: First, ERAP1 expression in two melanoma cell lines (Ma-Mel-86a, -86c) established from different metastasis of patient Ma-Mel-86 was confirmed by quantitative RT-PCR and Western Blot. By shRNA transfection of tumor cells, ERAP1 was downregulated. To determine if dowregulation of ERAP1 expression influences autologous T cell activation, bulk $CD8^+$ T lymphocytes from the peripheral blood were stimulated with ERAP1-shRNA transfected Ma-Mel-86 cells in comparison to control shRNA or empty vector transfected melanoma cells. T cell activation was analyzed by IFN-γ ELISA assay.

Results: IFN-γ ELISA assays, performed after 24 h coculture, revealed significantly impaired IFN-γ production of bulk $CD8^+$ T cells in the presence of ERAP1 knockdown Ma-Mel-86 cells.

To get knowledge about ERAP1-dependent tumor antigen epitopes, autologous $CD8^+$ T cell clones of known specificity were incubated with the indicated melanoma target cells. Three of four analyzed T cell clones were significantly less activated by ERAP1-knockdown melanoma cells.

Conclusions: ERAP1 activity in melanoma cells influences specific T cell activation via the amount of the generated epitopes suggesting that tumor cells might escape from efficient T cell activation by alterating ERAP1 expression.

P1327

Adaptive immunity contributes to leukemia progression by activating the Wnt-pathway in leukemia stem cells via the CD70-CD27-interaction

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Purpose/Objective: Chronic myelogenous leukemia (CML) is a myeloproliferative neoplasia that arises from leukemia stem cells (LSCs) harboring the oncogenic BCR/ABL translocation. Clinically, CML is characterized by a chronic phase that inevitably evolves into acute leukemia. During the chronic phase, a leukemia-specific immune response is thought to contribute to disease control, but whether the immune system can also promote the disease is unknown.

Materials and methods: Retroviral transduction of BCR/ABL-GFP into murine bone marrow (BM) cells, followed by transplantation into sublethally irradiated congenic recipients, was used to establish CML-like disease in mice. Sublethal irradiation (4.5 Gy) preserved the endogenous adaptive immune system of the recipients, allowing analysis of anti-leukemic immune responses.

Results: In the present study, we document that BCR/ABL^+ LSCs express the TNF receptor family member CD27. CD70-expressing T cells ligated CD27 on LSCs in the BM, which increased the expression of Wnt target genes in LSCs by enhancing nuclear localization of active β-catenin and the TRAF2- and NCK-interacting kinase (TNIK). This resulted in increased proliferation and differentiation of LSCs. Blocking CD27 signaling by transplanting LSCs from $CD27^{-/-}$ mice or by monoclonal antibody treatment delayed disease progression and prolonged survival. Furthermore, CD27 was expressed on $CD34^+$ cells in the bone marrow of CML patients and healthy controls, and CD27

signaling promoted growth of BCR/ABL$^+$ human leukemia cells by activating the Wnt pathway.
Conclusions: Here, we describe a novel signaling axis in leukemia, the CD27-TRAF2-TNIK-Wnt pathway. Activated immune cells promoted LSC proliferation and differentiation via the CD70-CD27-interaction. Our results reveal a novel mechanism by which adaptive immunity contributes to leukemia progression. Targeting CD27 on LSCs may represent an attractive therapeutic approach to block the Wnt/β-catenin pathway in leukemia.

P1328
B and T lymphocyte attenuator mediates inhibition of tumor-reactive CD8+ T cells in patients after allogeneic stem cell transplantation

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Purpose/Objective: Allogeneic stem cell transplantation (alloSCT) can cure hematological malignancies by inducing alloreactive T cell responses targeting minor histocompatibility antigens (MiHA) expressed on malignant cells. Despite induction of robust MiHA-specific T cell responses and long-term persistence of alloreactive memory T cells specific for the tumor, often these T cells fail to respond efficiently to tumor relapse. Previously, we demonstrated the involvement of the coinhibitory receptor programmed death-1 (PD-1) in suppressing MiHA-specific CD8$^+$ T cell immunity. In this study, we investigated whether B and T lymphocyte attenuator (BTLA) plays a similar role in functional impairment of MiHA-specific T cells after alloSCT.
Materials and methods: In order to examine the role of BTLA and PD-1 in T cell dysfunction after alloSCT, we analyzed T cells from transplanted patients and healthy donor. Furthermore, we stained tumor cells for ligands. Importantly, we performed *ex vivo* stimulation assays with MiHA-specific T cells in the presence of anti-BTLA and/or anti-PD-1 blocking antibodies (Ab). Subsequently, cell proliferation, IFN-γ and TNF-α secretion and degranulation upon Ag challenge was investigated.
Results: In addition to PD-1, we observed higher BTLA expression on MiHA-specific CD8$^+$ T cells compared with that of the total population of CD8$^+$ effector-memory T cells. In addition, BTLA's ligand, herpes virus entry mediator (HVEM), was found constitutively expressed by myeloid leukemia, B cell lymphoma, and multiple myeloma cells. Interference with the BTLA-HVEM pathway, using a BTLA blocking Ab, augmented proliferation of BTLA$^+$PD-1$^+$ MiHA-specific CD8$^+$ T cells by HVEM-expressing dendritic cells. Notably, we demonstrated that blocking of BTLA or PD-1 enhanced *ex vivo* proliferation of MiHA-specific CD8$^+$ T cells in respectively 7 and 9 of 11 allo-SCT patients. Notably, in 3 of 11 patients, the effect of BTLA blockade was more prominent than that of PD-1 blockade. Furthermore, these expanded MiHA-specific CD8$^+$ T cells competently produced effector cytokines and degranulated upon Ag reencounter.
Conclusions: Together, these results demonstrate that BTLA-HVEM interactions impair MiHA-specific T cell functionality, providing a rationale for interfering with BTLA signaling in post-stem cell transplantation therapies.

P1329
B-1 cells expressing CCR5 can modulate the development of murine melanoma

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Purpose/Objective: Melanoma is a type of skin cancer which can acquire invasive behavior. In the tumor microenviroment, inflammatory cells can influence almost every aspects of cancer progression, but the knowledge on the cellular and molecular mechanism are not completely clarified. Studies have attributed an important role to B-1 cells, a subset of B lymphocytes, in melanoma progression. Objective: The aim of this work was evaluate the influence of CCR5 expressed in B-1 cells on the course of murine melanoma progression.
Materials and methods: Experimental metastasis assay were performed by injecting intravenously 1x10^5 B16 melanoma cells in C57Bl/6, CCR5$^{-/-}$ and CCR5$^{-/-}$ that received adoptive transfer of B-1 cells from C57Bl/6. After 14 days, lung tumoral colonies were counted in each group. The same experimental groups were used to evaluated subcutaneous tumor growth and survival rate.
Results: The present study demonstrated that B16 cells release soluble factors that are chemoattractant for B-1 cells. It was demonstrated that 10% of peritoneal B-1 cells express the chemokine receptor CCR5 on their surface. These cells, when inoculated into animals CCR5$^{-/-}$, led to 10-fold decrease in the number of metastatic nodules. Moreover, this approach reduced subcutaneous tumor growth maintaining a stable volume of tumor whereas control animal presented exponential tumor growth. In addition, this treatment significantly increased the survival of the animals.
Conclusions: Our data suggest that B-1 cells expressing CCR5 can modulate the development of murine melanoma. So, this work may contribute to a better understanding of B-1 cells chemotaxis and the influence of migration in evolution of melanoma.

P1331
CCR2-dependent VEGF-A production by infiltrating macrophages sustains Kaposi sarcoma growth

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Purpose/Objective: Inflammatory chemokines are overexpressed in several tumors where they exert different protumoral functions such as monocyte recruitment at tumor site, tumor-associated macrophage polarization and promotion of metastasis. Here we want to assess the involvement of the atypical chemokine receptor D6, that drives to degradation several inflammatory CC chemokines, in a cancer model.
Materials and methods: An *in vivo* model of Kaposi sarcoma was performed using a cell line (KS-IMM) overexpressing the atypical chemokine receptor D6 injected subcutaneously in nude mice.
Results: D6 overexpression did not influence KS-IMM *in vitro* proliferation rate while significantly reduced its *in vivo* growth when compared to mock cells. Leukocyte infiltrate of D6 overexpressing tumors was mainly composed by Ly6C^{high}/F480$^+$ monocytes with strong reduction of both neutrophils and tumor-associated macro-

phages when compared to mock tumors. D6 overexpressing tumors had decreased amount of mVEGF-A and reduced angiogenesis compared to mock tumors. The use of a CCR2 antagonist reduced monocyte recruitment at tumor site, impaired their differentiation to TAM and inhibited tumor growth. KS-IMM conditioned medium enhanced VEGF-A production by bone marrow-derived macrophages with a CCR2 and COX_2 dependent mechanism.
Conclusions: The regulation of CCR2 ligands bioavailability by D6 overexpression at tumor site inhibits monocyte differentiation and VEGF-A production resulting in reduced tumor growth.

P1332
CD27-signaling increases the frequency of regulatory T cells and promotes tumor growth

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Purpose/Objective: Signaling of the TNF-receptor superfamily member CD27 activates costimulatory pathways to initiate T and B cell responses. CD27-signaling is regulated by the expression of its unique ligand CD70 on subsets of activated immune cells. In this study, we analyzed the role of the CD27-CD70-interaction in the immunological control of solid tumors.
Materials and methods: To investigate the role of CD27-signaling in tumor growth in the context of an already established tumor stroma, we transplanted solid fragments of MC57 fibrosarcoma s.c. into the flanks of wild-type and CD27-deficient mice.
Results: In tumor-bearing wild-type mice, the CD27-CD70-interaction reduced apoptosis of intratumoral regulatory T cells (Tregs). This resulted in an increase ofTreg frequencies, reduction of tumor-specific T cell responses, increase in angiogenesis and in promotion of tumor growth. Consequently, the frequency of Tregs and growth of solid tumors were reduced in CD27-deficient mice or in wild-type mice treated with the CD70 blocking monoclonal antibody FR70. CD27-signaling induced $CD4^+$ effector T cells to produce interleukin-2, a key survival factor for Tregs. Furthermore, in patients with non-small cell lung cancer (NSCLC), the serum concentration of soluble CD27, a marker of the CD27-CD70 interaction *in vivo*, correlated with poor prognosis.
Conclusions: Our findings therefore provide a novel mechanism by which the adaptive immune system enhances tumor growth. In combination with other therapeutic regimens, blocking CD27-signaling is a promising approach to reduce Treg formation in the tumor environment and to improve tumor control.

P1334
Changes in TGF-beta concentration in bone marrow plasma in the course of common acute lymphoblastic leukemia (cALL) treatment

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Purpose/Objective: Transforming growth factor β is a multipotent cytokine with a great impact on cellular homeostasis. It plays an important role in cellular differentiation, senescence, regulation of cell growth, migration and apoptosis. These actions determine TGF β influence on oncogenesis, progression and metastasis. TGF β signaling pathway has a defined role in regulation of normal hematopoesis and is frequently impaired in hematologic malignancies. In same types of leukemia, blastic cells demonstrate decreased sensitivity to TGF β action. It may be connected with a lack of TBR-I and TBR-II receptors.

The aim of this study was the evaluation of TGF β concentration in bone marrow plasma in the course of common limfoblastic B leukemia treatment.
Materials and methods: 46 children with cALL, hospitalized in The Department of Pediatrics, Hematology and Oncology Medical University of Warsaw, were enrolled to the study group. The concentration of TGF β was measured using immunoenzymatic ELISA technique in the bone marrow plasma before and in 15, 33 and 72 day of treatment.
Results: Results of our analyses revealed significant difference in TGF β concentration before introduction of the therapy and in subsequent (15, 33, 72) days of treatment. Statistical significance reached the value $P < 0.0001$ in each case. Differences were also found between subsequent days when compared. The median of TGF β in 0, 15, 33 and 72 day was of: 3180.355pg/ml; 5444.645pg/ml; 11451.5pg/ml and 9645.78pg/ml respectively.
Conclusions: Gradual increase of TGF β concentration up to the 33 day and decrease in 72 day observed in the course of treatment confirm essential role of transforming growth factor in cellular proliferation and apoptosis. It suggests also possible influence of this cytokine on hematopoesis. However influence of chemotherapy on TGF β secretion has to be further evaluated.

P1335
Chemotherapy by dacarbazine improves antitumor immunity induced by a DNA vaccine targeting telomerase

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Purpose/Objective: Given its widespread expression in cancers, telomerase reverse transcriptase (TERT) is an attractive target for cancer vaccine. Recently it has been shown that chemotherapy can kill cancer cells by causing them to elicit an immune response or alternatively, by interfering with host immunity to promote immune attack. In this study we evaluated TERT-based DNA vaccine combined to immunomodulating chemotherapy.
Materials and methods: A mutated TERT DNA vaccine encoding a non-functional telomerase was used in HLA-A2/DR1 transgenic mice bearing B16-HLA-A2 melanoma. Vaccine was combined to Dacarbazine (DTIC), a reference chemotherapy used in advanced melanoma.
Results: TERT DNA vaccine induces T cell responses against multiple TERT-derived CD4 and CD8 epitopes *in vivo*. These responses are strongly enhanced by the association of DTIC to the vaccine and effectively eradicates B16-HLA-A2 tumor growth in mice. An increased level of activated immune cells (dendritic cells, NK cellsÉ) was found in mice treated by DTIC. Furthermore, mice treated by vaccine plus DTIC exhibit high frequency of functional tumor-infiltrative lymphocytes (TILs).
Conclusions: In this study, we showed that DTIC chemotherapy combined with TERT-based vaccine significantly increases TERT-specific T cell responses and provides efficient tumor protection in mice. Altogether these results underline the interest of combining TERT-based DNA vaccine to immunostimulating chemotherapies.

P1336

Chronic hepatitis B co-infection, with hepatitis C and detection of carcinogenic markers

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Purpose/Objective: Thedetection of occult HBV infection in chronic hepatitis C patients using qualitative PCR. Detection of $CD45^{-}$ $CD90^{+}$, as a biomarker in hepatocellular carcinoma patients.
Materials and methods: From 30 patients suffering from HCVdisease, samplescollected and histopathologically verified, 10 healthy control subjects. Patientsclassified into two groups according to serological tests for viral hepatitis markers (HBsAg, HCV-ab), the clinical findings, laboratory findings, and alpha-fetoprotein. Total DNAextracted from 100ul of serum. Samples assayed for HBVusing three PCR assays with primer pairs, their sequences were taken from the surface, core, and X-genes of the viral genome. CD90 purified and analysed by flow cytometry.
Results: Out of forty patients,30 had serological positive HCV infection, all negative for HBsAg. Using PCR, off 4 0 patients, seranine were positive for HBV DNA (15%), byPCR assays, documentingoccult HBV infection. Fivesamples, positive for HBV DNA (surface gene) (12.5%), three werepositive for HBV, DNA (X gene) (7.5%), andone patient samplepositive HBV DNA (core gene) (2.5%). Two fromnine positive samples were positive for both X-gene and surface gene. In 5 of 15 cases more than 0.2%in liver cirrhosis, one case only 0.1%, in healthy group expression is 0%.
Conclusions: data suggest that occult HBVinfection may have clinical significance in chronic hepatitis C patients.

P1339

Composition, organization and clinical impact of the adaptive and innate immune microenvironments in lung metastases from colorectal and renal cell carcinoma

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Purpose/Objective: It is now well established that the immune microenvironment of primary tumors impacts clinical outcome. During cancer progression, cancer cells undergo genetic alterations and the composition and organization of the immune microenvironment may differ between metastatic and primary tumors. Moreover, it could be expected that if metastatic cells underwent immunoediting and escaped immunosurveillance, immune infiltrates in metastases might not be associated with clinical outcome.
Materials and methods: Human lung metastatic colorectal (CRC-MET, $n = 140$), lung metastatic renal cell carcinoma (RCC-MET, $n = 52$), primary CRC ($n = 12$) and primary RCC ($n = 14$) tumors were analyzed for immune cell infiltrates (CD3+ and CD8+ T cells, mature dendritic cells (mDCs) and natural killer (NK) cells) using immunohistochemistry techniques. qPCR was implemented to investigate the gene expression profiles related to immune populations and their functions in lung metastases from both CRC ($n = 19$) and RCC ($n = 13$).
Results: We show for the first time that lung metastases from CRC and RCC have different immune infiltrates. Elevated mDCs ($P < 0.0001$) and reduced densities of NK cells ($P < 0.0001$) were observed in CRC-MET as compared to RCC-MET, whereas densities of CD3+ and CD8+ T cells were similar in both metastases. Interestingly, the densities of immune cells correlated significantly ($R > 0.6$, $P < 0.05$ for all comparisons) between coincident or relapsing metastases and between metastases and primary tumors from the same patient. This indicates that tumor cells induce a characteristic and reproducible immune pattern in the primary tumor and associated metastases. A high infiltration of CD8+ T cells and mDCs was a good prognosticator of prolonged survival in CRC-MET ($P = 0.008$), whereas it is associated with poor survival in RCC-MET ($P < 0.0001$). A high density of NK cells was associated with longer survival in metastatic RCC patients ($P = 0.002$). A Th1 orientation was found in CRC-MET whereas a more complex immune genes profile with a Th1 orientation mixed with genes linked to Th2, inflammation and immunosuppression were detected in RCC-MET.
Conclusions: Our findings revisit the 'seed and soil' theory and highlight the role of the tumor cell in shaping the tumor-associated immune microenvironment. The immune signature appears to be a phenotypic marker for the disease and is remarkably reproduced between primary and metastatic sites in the same patient. It is the most powerful prognostic marker for overall survival in lung metastases from CRC and RCC.

P1341

Cross-talk between p53 and CXCR5 in breast carcinoma cells

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Purpose/Objective: Elevated expression of chemokine receptors by tumors has been reported in many instances and is related to a number of survival advantages for tumor cells including abnormal activation of prosurvival intracellular pathway. The objective of this study was to see whether impairment of p53 function may be directly related to expression of chemokines and their receptors by tumor cells.
Materials and methods: p53 expression in MCF-7 cells was suppressed by lentiviral expression of an shRNA for p53. Chemotaxis was assessed using agarose spot assay.
Results: The suppression of p53 expression led to increased expression of CXCR5 and to increased cell chemotaxis along CXCL13 gradient.
Conclusions: We have established the relationship between the expression of tumor suppressor p53 and the chemokine receptor CXCR5 in breast cancer cells MCF-7. Transcriptome changes in p53-supressed MCF-7 cells and possible direct role of p53 in CXCR5 gene regulation are under investigation. The study was supported by grant 10-04-92657 from Russsian Foundation for Basic Research and by contract 16.740.11.0006 with the Ministry of Education and Science of the Russian Federation.

P1342

Cytokine Induced Killer cells immunotherapy overcomes the resistance to MET tyrosine kinase inhibitors in nonsmall cell lung cancer

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Purpose/Objective: Prognosis for late stage lung cancer under conventional therapies remains poor. We hereby investigated whether immunotherapy approach with CIK (Cytokine Induced Killer) cells might overcome acquired resistance to MET tyrosine kinase inhibitors

(TKI) in NSCLC. CIK cells are a subset of *ex -vivo* expanded T lymphocytes with T-NK phenotype and MHC-unrestricted antitumor activity which is mediated by the interaction of CIK's membrane receptor NKG2D with MIC A/B and ULBPs ligands expressed on the tumor cells. We hypothesized that acquired resistance and consequent acquisition of more aggressive characteristics of NSCLC cells might be associated with upregulation of MIC A/B and ULBPs ligands.

Materials and methods: MET inhibitor resistant cells are generated by treating MET -addicted cells with increasing concentrations of the MET small-molecule inhibitors PHA-665752 OR JNJ38877605. CIKs were expanded from 10 healty donors, starting from PBMC, with the timed addition of IFN-gamma, Ab anti-CD3 and IL2. Cytotoxicity assays were preformed to detect antitumor activity of CIK cells.

Results: The median expansion of CIK cells was 29-fold (range, 7−43). Expanded CIKs included a median of 39% $CD3^+$ CD56+ cells (range, 21−74). Median expression of NKG2D receptor was 87% (range, 60−95). NSCLC cells sensitive to MET TKI (EBC 1 WT) had 51% membrane expression of MIC A/B. Such expression was upregulated on the cells that acquired resistance (EBC 1R125) reaching 100%. There was no difference observed in ULBPs ligands expression between both cell lines. CIK cells efficiently killed both, sensitive and resistant cell lines. Specific killing was significantly higher against resistant cells comparing to sensitive cells. An average specific killing of resistant line was 55%, 51%, 43% and 37% at 40:1, 20:1, 10:1 and 5:1 effector/target ratio respectively. The sensitive line had, at the same ratio, an average specific killing of 38%, 34%, 26%, 23%, respectively ($n = 14$).

Conclusions: Our data describe for the first time the association of MIC A/B upregulation and MET TKI resistance in NSCLC. Acquired resistance is a common and limiting problem for chronic therapy with many TKI, including MET TKI. CIK cells adoptive immunotherapy might address this issue targeting MET TKI resistant tumor cells.

P1343

Cytotoxic T cells induce proliferation of chronic myeloid leukemia stem cells by secreting interferon-y

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Purpose/Objective: Chronic myelogenous leukemia (CML) is a clonal myeloproliferative neoplasia arising from the oncogenic BCR/ABL translocation in hematopoietic stem cells (HSCs), resulting in a leukemia stem cell (LSC). Cure of CML depends on the eradication of LSCs. Unfortunately, LSCs are resistant against current treatment strategies. The host's immune system is thought to contribute to disease control and several immunotherapy strategies are under investigation. However, the interaction of the immune system with LSCs is poorly defined. Therefore, we analyzed the interaction of leukemia-specific $CD8^+$ effector cytotoxic T cells (CTLs) with LSCs.

Materials and methods: In order to analyze the interaction of CTLs with defined antigen specificity with LSCs, we used donor BM of H8 transgenic mice ubiquitously expressing the LCMV glycoprotein epitope gp33 under the control of an MHC class I promoter to generate a CML in BL/6 mice (H8 CML). In this setting, BCR/ABL-GFP-expressing cells co-express the LCMV-gp33 antigen. However, results may differ if a relevant leukemia antigen is targeted. Therefore, we additionally induced CML using HLA-A2tg mice. In this setting, BCR/ABL-GFP-expressing cells co-express the BCR/ABL-derived junctional peptide b3a2 on HLA-A2.1.

Results: In the present study, we document that LSCs express major histocompatibility complex (MHC) and costimulatory molecules and are recognized and killed by specific CTLs *in vitro*. In contrast, therapeutic infusions of effector CTLs to CML mice *in vivo* failed to eradicate LSCs but paradoxically increased LSC numbers. LSC proliferation and differentiation was induced by CTL-secreted interferon (IFN)-γ. Effector CTLs were only able to eliminate LSCs in a situation with minimal leukemia load where CTL-secreted IFN-γ levels were low.

Conclusions: Our study reveals a novel mechanism by which the immune system contributes to leukemia progression and may be important to improve T-cell-based immunotherapy against leukemia.

P1344

Dendritic cell editing by activated natural killer cells results in a more protective cancer-specific immune response

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Purpose/Objective: Over the last decade, several studies have extensively reported that activated natural killer (NK) cells can kill autologous immature dendritic cells (DCs) *in vitro*, whereas they spare fully activated DCs. This led to the proposal that activated NK cells might select a more immunogenic subset of DCs during a protective immune response. However, there is no demonstration that autologous DC killing by NK cells is an event occurring *in vivo* and, consequently, the functional relevance of this killing remained elusive. Here we aimed to elucidate whether NK cell-mediated DC killing indeed takes place *in vivo* and investigate its possible role on antigen specific immune response.

Materials and methods: NK cells were activated in a mouse model by injecting MHC-devoid cells. Draining lymph nodes were collected and lymph node DCs functions analyzed. Finally, in a model of anti-cancer vaccination, the functional relevance of DC editing by NK cells was investigated.

Results: A significant decrease of $CD11c^+$ DCs was observed in draining lymph nodes of mice inoculated with MHC-devoid cells. Residual lymph node DCs displayed an improved capability to induce T cell proliferation. In addition, during anti-cancer vaccination, the administration of MHC-devoid cells together with tumor cells increased the number of tumor-specific CTLs and resulted in a significant increase in survival of mice upon challenge with a lethal dose of tumor cells. Depletion of NK cells or the use of perforin knockout mice strongly decreased the tumor-specific CTL expansion and its protective role against tumor cell challenge.

Conclusions: Our data indicate that NK cell-mediated DC killing takes place *in vivo* and is able to promote expansion of cancer-specific CTLs. The results also show that cancer vaccines could be improved by strategies aimed at activating NK cells.

P1345

Density of tertiary lymphoid structures is associated with T lymphocyte infiltration and protective immune signature in human lung tumors

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Purpose/Objective: An important number of studies have demonstrated a strong correlation between density of tumor infiltrating T

cells (TIL) and clinical outcome in several types of human solid cancers. Nevertheless, the mechanisms governing T-cell infiltration and activation into tumors remain poorly characterized. In lung cancer, our team has observed the presence of tertiary lymphoid structures called Ti-BALT (Tumor-induced Bronchus-Associated Lymphoid Tissues). These structures present features of an ongoing immune response and their density is associated with long-term survival for lung cancer patients, suggesting their implication in local T cell recruitment and activation. Our aim was to study the influence of these structures on the infiltration, composition, density and functionality of the immune infiltrate in lung tumors.

Materials and methods: The expression of relevant molecules was assessed on fresh TIL by multicolor flow cytometry, on tissue sections by immunohistochemistry and on frozen tumors by Low Density Array analysis.

Results: We identified presence of intra-tumoral PNAd$^+$ high endothelial venules (HEVs) exclusively associated with Ti-BALT. We also report that CD62L$^+$, the PNAd ligand, is specifically expressed on Ti-BALT T cells. Moreover, a specific chemoattractant gene expression signature associated with T cell presence was identified in Ti-BALT, which strongly argue for an active recruitment of CD62L$^+$ T cells from HEVs to Ti-BALT. We also observed that TIL located in Ti-BALT had a naïve and early memory T cell phenotype, as observed in canonical secondary lymphoid organs. Moreover, we detected presence of DC-Lamp$^+$ mature dendritic cells in Ti-BALT, which could be in close contact with Ki-67$^+$ proliferating TIL, suggesting a local priming of T cells in these structures. Finally, Ti-BALT density was associated with increased activated effector-memory T cell infiltration and upregulation of genes related to T-helper 1 adaptive immunity and T-cell cytotoxicity.

Conclusions: All Together, these data suggest that Ti-BALT represent a privileged area for T cell recruitment and activation in the primary site of the tumor. This mechanism could lead to the establishment and maintenance of a protective anti-tumor immune response directly in the tumor.

P1346

Differences of myeloid cell subsets in a chemically induced rat breast carcinoma model

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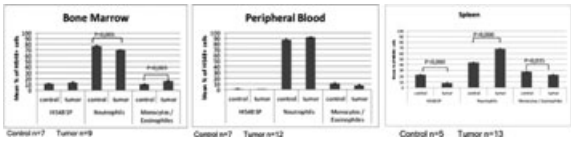
Purpose/Objective: The aim of this study is to identify the distribution of the myeloid cells at different immune compartments in a rat breast cancer model. In addition, we intend to reveal novel myeloid derived suppressor cell subsets in rats. At the first stage of our study, amounts and distributions of myeloid cell populations were determined.

Materials and Methods: Four injections of *N*-Methyl N-Nitrosourea (NMU) were performed to 21 days old female Sprague−Dawley rats at a dose of 50 mg/kg/week and mammary adenocarcinoma development was observed in about 40 days post injection. Following sacrifications, cells were isolated employing erythrocyte lysis from the spleen, peripheral blood and bone marrow tissues of tumor bearing and healthy control animals. CD11b/c, HIS48, CD172a, HIS36, CD103, RP1, CD80, CD86, CD40, RT1B (MHC-II) and CD161 antibodies were used for staining and data acquisition was performed by FACS ARIA II flow cytometer. Kaluza and SPSS softwares were used to analyse the data and Mann−Whitney *U*-tests were used to determine statistical significances.

Results: Rp-1$^+$ cells defining Neutrophils are found to be co-expressing HIS48 target and CD11b/c at all tissues. The number of neutrophils were diminished at tumor bearing animals' bone marrow ($P = 0.001$), whereas HIS48$^+$, CD11b/c$^+$, Rp-1$^-$ monocytes numbers were elevated respectively ($P = 0.003$). The most dramatic difference was observed in tumor bearing rats' spleen. At this tissue, the accumulation of neutrophils at tumor bearing rats was apparent ($P = 0.000$), being complementary, HIS48 single positive cells and monocytes were slightly lower at tumor bearing rats' spleen ($P = 0.000$, $P = 0.035$). In addition, macrophage numbers do not differ between control and tumor bearing rats at any tissue. Rp-1$^+$ cells of spleen origin have also been found to suppress PHA induced CD4$^+$ T cell proliferation of control splenocytes.



Conclusions: The dramatic increase detected in neutrophil population at spleen might be related to premature migration of these cells from bone marrow of tumor bearing animals. Furthermore, the remarkable decrease of HIS48 single positive cells and the elevation of neutrophil numbers at spleen might be related with a distorted maturation process among these cells in this microenvironment

P1347

Dominant and cryptic CD4 T cell epitopes reside in the signal peptide of the tumor-shared antigen Midkine

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Purpose/Objective: We have previously shown that the angiogenic factor Midkine (MDK) induced HLA-A2 restricted and tumor-specific CTL responses. Because of its wide expression in malignant cells and its vaccine potential, we investigated the CD4 T cell response specific for MDK.

Materials and methods: By weekly stimulations of T lymphocytes harvested in HLA-DR-typed healthy donors, we derived CD4 T cell lines specific for several Midkine peptides.

Results: Most of the T cell lines reacted with the peptides 3 and 4, which were located in the MDK signal peptide and overlapping with it, respectively. Accordingly, the MDK signal peptide appears as especially rich in good binders to the most frequent HLA-DR molecules in Europe. Peptide 3-specific T cell lines were specifically stimulated by autologous dendritic cells loaded with lysates of MDK-transfected cells or of tumor cells naturally expressing MDK. One T cell line was stimulated by HLA-compatible MDK-transfected tumor cells. In contrast, the peptide 4-specific T cell lines were not stimulated in these conditions.

Conclusions: Peptide 4 did not seem therefore to be displayed at the surface of the APC from MDK expressed in tumor cells. It might be cleaved during the MDK processing. This peptide behaves as a cryptic epitope since it does not elicit a MDK-specific T cell response but a peptide-specific response, only. We therefore conclude that peptide 3, which is present in the signal peptide of MDK leads to a tumor-specific CD4 T cell response demonstrating that signal peptide can be accessible to recognition by CD4 T cells, while peptide 4, which overlaps the joining region with the signal peptide, is not. Our data illustrate how CD4 T cell epitopes residing in the signal peptide could contribute to tumor immunity.

P1348

Effective anti-tumor vaccination by CIITA-transfected tumor cells as result of optimal CD4+ T helper cell stimulation

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Purpose/Objective: Most of the poor protection of present vaccination approaches against cancer is due to inefficient stimulation of MHC class II-restricted CD4+ T helper cells (TH). Thus newly conceived anti-tumor vaccination strategies based on optimal stimulation of TH cells are urgently needed.
Materials and methods: Murine tumor cell lines of different histotype were stably transfected with the MHC-II transactivator CIITA and CIITA-dependent MHC-II-positive cells were isolated by cell sorting. Immunocompetent mice were injected with CIITA-tumor cells or with parental cells. Immune response against the tumor was evaluated by: (1) the kinetics of tumor growth *in vivo*, (2) the anamnestic response to a parental tumor cell challenge; (3) the anti-tumor specificity of distinct lymphocyte subpopulations evaluated by *in vitro* and *in vivo* transfer assays; (4) the characterization of the tumor microenvironment and infiltrate by immunohistochemistry.
Results: Tumor rejection and/or retardation of growth was found for CIITA-transfected mammary, colon, renal carcinomas and for sarcomas. Animals rejecting CIITA-transfected tumors acquired specific immunological memory as demonstrated by resistance to challenge with untransfected parental tumors. Adoptive cell transfer experiments demonstrated that tumor immunity was the result of optimal priming of CD4+ T cells and consequent triggering of CD8+ T lymphocytes. Importantly, CIITA-tumors were rapidly infiltrated by TH cells and subsequently by dendritic cells and CD8+ T cells. T cells from CIITA-tumor vaccinated mice used in an adoptive immunotherapy model showed the cure at early stages and significantly prolonged survival at later stages of tumor progression. Importantly, CD4+ T cells were clearly superior to CD8+ T cells in anti-tumor protective function. Interestingly, the protective phenotype was associated to both a Th1 and Th2 polarization of the immune effectors and to reduction of Tregs in spleen and draining lymphonodes.
Conclusions: These results establish the general application of our tumor vaccine model as a superior model to induce anti-tumor $CD4^+$ TH, key cells for triggering anti-tumor effector mechanisms, and disclose the additional application of this strategy for producing better lymphocyte effectors for adoptive anti-tumor immunotherapy.

P1349

Effects of the hypoxia-inducible factors inhibitor 'topotecan' on the suppressive activity of tumor-associated myeloid cells

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Purpose/Objective: Areas of low oxygen levels (hypoxia) are frequently found in solid tumors. Hypoxia has an important impact on myeloid cells with opposite effects on the innate and adaptive immunity. Hypoxia promotes the recruitment, activation and survival of innate immunity while inhibiting the adaptive immunity. Responses to hypoxia are mostly orchestrated by activation ofHypoxia-Inducible Factors (HIFs), which represent suitable targets for cancer therapy. Several HIF inhibitors have been identified. Among them Topotecan (TPT), a topoisomerase I inhibitor, is currently used in clinic for the treatment of ovarian cancer|, small-cell lung cancer| and cervical cancer|. Noteworthy, tumor development is also paralleled by a strong expansion of suppressor myeloid cell populations, including both suppressor dendritic cells (DC) and myeloid-derived suppressor cells (MDSC). Here, we evaluated whether targeting HIFs activation in both DC and MDSC may be an integrant part of the therapeutic activity of HIFs inhibitors, including TPT.
Materials and methods: Cell culture.

DCs were differentiated *in vitro* from either peripheral blood monocytes of healthy human donors or mouse bone marrow cells. MDSCs were differentiated *in vitro* from bone marrow too.

MLR.

Purified allogeneic T-cells or OVA-specific splenocytes were added to graded doses of DCs or MDSCs in 96-well plates. T cells proliferation was assessed by Thymidine incorporation.

ELISA.

Cytokine levels were measured using Duo-Set kits (R&D).
Results: On both mouse and human DCs, TPT displays an immunogenic effect by up-regulating their *in vivo* production of IL-1β and IL-12, associated with an increased Th1response (increased T cell-mediated Interferon-g production in draining lymph nodes). On the contrary, TPT restrains the immunosuppressive capacity of MDSCs by reducing both their NO production and capacity to inhibit T cell proliferation *in vitro*. These effects of TPT on DC and MDSC functions were observed in both hypoxic and normoxic conditions and appear, at least partially, independent from HIFs activation.
Conclusions: Our data indicate a possible effect of TPT as immune-stimulant drug. We wish to confirm these preliminary results *in vivo* and to establish the potential use of TPT in a combined chemotherapeutic/adjuvant preclinical approach.

P1350

Establishment and characterization of a tumor cell line from bone marrow of patient with myelodysplastic syndrome

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Purpose/Objective: Myelodysplastic syndrome (MDS) that ineffectively produces myeloid class of blood cells is formerly known as preleukemia. Many patients with MDS are at risk of developing leukemia. However, the mechanism of cells from MDS transformed into leukemia is unclear. To study the tumorigensis of cells form MDS, we established and characterized a tumor cell line from the bone marrow of patient with MDS.
Materials and methods: A tumor cell line, BML01, was established from the bone marrow of patient with MDS. To determine the tumorigenecity of BML01, the cells were implanted into the DOS-SCID mice and cultured in serum-reduced culture medium *in vitro*. To define the surface marker expression of the cells, many different kinds of specific antibodies were used to stain and analyses by flowcytometry. And also, the tumor cells were stimulated with PHA, LPS, mitomycin or IL-2 to test the characteristics of cells.
Results: The BML01 cells are of a hematopoietic cell as demonstrated by the expression of CD45 surface marker. BML01 cells posses characteristics of tumor cells based on the cell are tumorigenic in NOD-SCID mice and reduced serum requirements for *in vitro* cultivation. The doubling time of BML01 cell is 16 h, and the cells have been successfully cultured *in vitro* for more than 50 generations. In addition, FACs analysis shows the BML01 cells also express surface molecules including CD14, CD30, CD44, CD81, CD107a, CD135, CD138, CD146, CD235 and CD274. The result of Liu's stain for the BML01 suggested the cells are mononuclear. Furthermore, the cells can be stimulated with PHA but not LPS, mitomycin or IL-2 in terms of the proliferation assay.
Conclusions: The new cell line provides us an *in vitro* system for the study on the oncogenesis of leukemia.

P1351

Ethanol effects MICA/B protein expression and release from the cells

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Purpose/Objective: MICA and MICB proteins (MHC class I chain-related proteins A/B) participate in regulation of immune reactions during tumorogenesis and some infections. The basic mechanism of MICA/B functioning is the interaction of MICA/B with the activating receptor of cytotoxic lymphocytes NKG2D. Recognition of MICA/B exhibited on the cellular surface leads to increase of cytotoxic activity of NK and T cells and the subsequent liquidation of the affected cell. At the same time, the circulating MICA/B forms releasing from the cells is capable to reduce the cytotoxic immune answer.

The aim of the work was to analyze effects of ethanol on MICA/B surface expression and shedding in several hemopoietic tumor cell lines with different levels of MICA/B surface expression.

Materials and methods: Surface expression of MICA/B was analyzed by flow cytometry and confocal microscopy. Intracellular proteins and colocalization were registered by confocal microscopy. Soluble MICA/B forms were detected by ELISA. Cells were incubated with ethanol for 18 h and then stained with fluorescent-labeled antibodies.

Results: The surface expression of MICA/B increased under the influence of ethanol (100–400 mM) in all types of investigated cells. Alcohol induced also MIC protein moving from the cytoplasm to the cell surface and releasing from cells in different forms. In normal conditions at the cell membrane there was a considerable level of colocalization of MIC protein with HSP70 that was previously shown to be exposed within lipid rafts. Under the action of ethanol location of these proteins became less coordinated.

Conclusions: We suggested that ethanol resulted in membrane domain reorganization. After cholesterol removing by incubation with methyl-β-cyclodextrin, the release of MIC from cells increased, which may indicate a role of lipid rafts in keeping MIC on the plasma membrane.

P1353

Expression of claudin-10 on B-1 cells triggers the higher metastatic potential of b16 murine melanoma cells

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Purpose/Objective: B-1 cells comprise a small fraction of the B cell family found preferentially in peritoneal and pleural cavities. Considering that B-1 cells are one of the main sources of interleukin-10 (IL-10), we previously demonstrated that cocultivation of B16 melanoma cells with B-1 cells from C57BL/6 mice (wt), but not with C57BL/6 IL-10 knockout B-1 cells (IL-10KO), increases the metastatic potential of melanoma cells. However, the molecule expressed on B-1 wt cells able to affect the metastatic potential of B16 cells has not yet been fully addressed. Therefore, the aim of this work was to identify the molecule expressed by B-1 wt, but absent in B-1 IL-10KO cells, which triggers increased metastatic potential of melanoma cells.

Materials and methods: To investigate differential gene expression between B-1 cells from wt and IL-10KO mice, expression profiles were generated using the *Affymetrix GeneChip Mouse Genome 430 2.0 Array*. Results were validated by western blot analysis and interference RNA assays were realized to evaluate biological function of differentially expressed molecules.

Results: Three independent experiments of microarrays analyses demonstrated differential mRNA expression of seven (7) genes between wt and IL-10KO B-1 cells. Among these genes, claudin-10, involved with cell communication and cell adhesion, was upregulated in B-1 wt cells. Data was confirmed by western blot analysis and interference RNA assays were performed to evaluate the biological function of claudin-10 in this model. Interestingly, silencing of claudin-10 expression on B-1 wt cells prevent their capacity to increase the metastatic potential of melanoma cells.

Conclusions: These results suggest that claudin-10 is one of the molecules present on B-1 cells responsible for the interaction that triggers the phenotypic changes of melanoma cells during coculture.

P1354

Functional analysis of neutrophils cultured in supernatant of CXCL7 overexpressing lung cancer cells

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Purpose/Objective: CXCL7 is a chemoatractant for the effector cells of innate immune system, neutrophils. The objective of this study is to analyze the function of neutrophils cultured in supernatant of lung cancer cells (Lewis Lung Carcinoma cells, LLC1) transfected with pCXCL7-IRES2EGFP plasmid.

Materials and methods: CXCL7 gene was cloned into the pIRES2EGFP vector. LLC1 cells were transfected by liposomal transfection agent. Stable CXCL7 overexpression of LLC1 cells was confirmed by using RT-PCR and ELISA. C57Bl6 mouse neutrophils were sorted from bone marrow by flow cytometry. Sorted neutrophils were cultured in supernatants of control or CXCL7 gene modified LLC1 cells. ROS production and phagocytic activity of neutrophils were evaluated by flow cytometry. Chemotactic activity of neutrophils was analysed using transwell insert system.

Results: Supernatant of CXCL7 gene modified LLC1 cells increased chemotaxis of neutrophils compared to the controls. ROS synthesis of neutrophils cultured in tumor supernatants was increased independent of CXCL7 gene modification. Phagocytic activity of neutrophils cultured in the presence of 10 ng/ml of recombinant CXCL7 was found to decrease.

Conclusions: CXCL7 gene modification affects neutrophil migration *in vitro*. CXCL7-gene modified LLC1 cells will be used for the establishment of LLC1 tumor in order to analyze immune responses against tumor *in vivo*.

P1357

Higher prevalence of LAP+ (latency TGFß-associated peptide) T cells in patients with early gastric cancer

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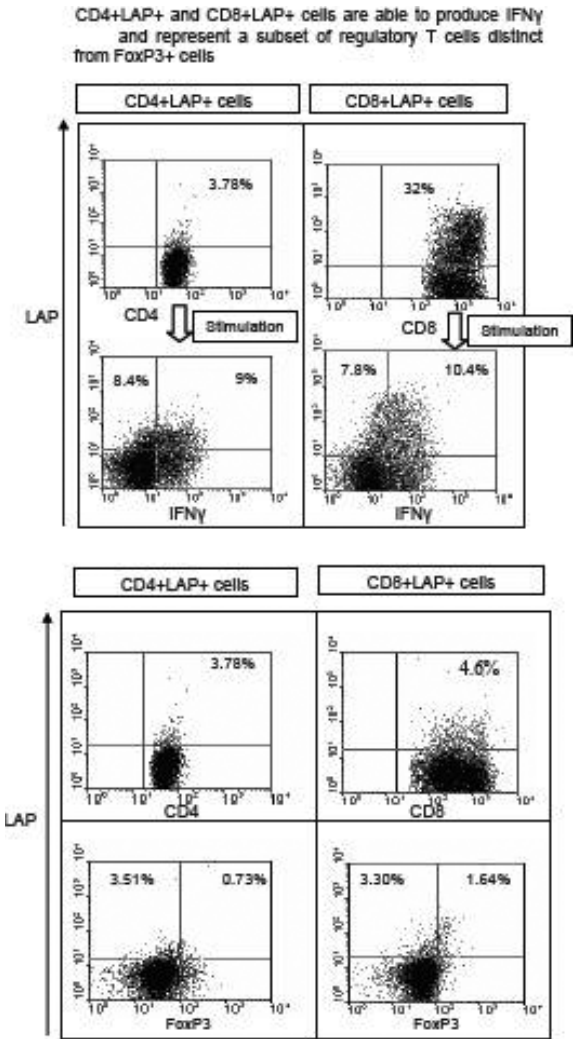
Purpose/Objective: The literature reports that regulatory T cells, identified as FoxP3+ cells, have a role in the progression of cancer. New immunoregulatory FoxP3-, TGFβ-producing cell subpopulations other than the well characterized FoxP3+ subset are now emerging. These subpopulations express the latency TGFβ-associated peptide (LAP) on their surface. In fact, two different immunoregulatory T-cell subpopulations have been recently described: CD4+LAP+ in humans

and CD8+LAP+ in a mice EAE model. Given the regulatory properties of these populations, we wished to analyze the prevalence of LAP+ T cells in gastric cancer patients.

Materials and methods: To this aim, enriched T cell populations were achieved from blood or gastric tissue (tumoral, TT or tumor-free, TF) samples from 22 patients, 6 with early (EGC) and 16 with advanced disease (AGC). Blood samples from 23 healthy control individuals (H) were also used. The expression of the CD4, CD8, LAP (inactive membrane-bound TGFβ) and FoxP3 markers was measured by cytometry. IFN-γ production by T cells upon stimulation was also measured by cytometry.

Results: The results reveal the presence of LAP+ T cells (both CD4+ and CD8+) subpopulations for the first time in cancer. CD8+LAP+ cells are increased in patients, especially in early cancer, in blood (H 2.8 ± 2.8%; EGC 7.1 ± 9.7%; AGC 4.9 ± 8.3%) and tumour samples (TF 3 ± 1%; EGC 6.6 ± 4.5%*; AGC 3.6 ± 3.1% *$P < 0.05$ versus TF). Likewise, the LAP+/CD8+LAP- ratio (a measure of the immunosuppression exerted on CTLs) is also increased in patients with early disease (blood: H 0.14 ± 0.14%; EGC 0.44 ± 0.6%; AGC 0.24 ± 0.4%, tissue: TF 0.23 ± 0.2%; EGC 0.37 ± 0.12%*, AGC 0.2 ± 0.3% *$P < 0.05$ versus AGC). Upon stimulation, these cells, different from the FoxP3+ cells, produce IFN-γ, a finding in keeping with previous published data stressing the role for IFN-γ production in the immunoregulatory function of LAP+ T-cells.



Conclusions: Results suggest that disease progression is accompanied by a decreased LAP membrane expression and, therefore, an increased LAP secretion for subsequent TGFβ activation, promoting tolerance to the tumour. LAP measurement in blood or tissue-derived T cells may be useful to evaluate the immune status of cancer patients.

P1358

Identification of posttranslationally modified tumour antigens in colorectal carcinoma reveals functional tumour-resident immunity targeting phosphoantigens

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Purpose/Objective: Multiple large studies have shown that an adaptive immune response, particularly infiltration of $CD8^+$ T cells, is associated with a positive clinical outcome in both primary and metastatic colorectal cancers (CRC). This suggests that there is a functional cytotoxic T cell response against the tumour. However, the tumour antigens that these $CD8^+$ tumour-infiltrating lymphocytes (TILs) are recognising have not been identified.

We have previously shown that posttranslationally modified peptides represent attractive tumour-specific antigens, as they are differentially expressed by tumour and normal tissues and may not be subject to central tolerance. Since dysregulation of signalling in cancers leads to aberrant phosphosphorylation, phosphorylated epitopes are a potential target.

Here, we identify CRC-associated phosphopeptides and assess the tumour-resident immunity against these novel antigens.

Materials and methods: CRC tumours and matched normal tissues were lysed, the MHC class I complexes affinity purified, and bound peptides eluted. Phosphopeptides were enriched using immobilised metal affinity chromatography and characterised using mass spectrometry. TILs, from the same tumours, were extracted and expanded, and their responses to the phosphopeptides assessed using ELISpot and multiplexed intracellular cytokine staining. Cytolytic activity was observed by staining for surface upregulation of CD107. Healthy donor PBMC responses were also assessed.

Results: We have identified over 20 tumour-specific, MHC class I associated phosphopeptides from CRCs. These include phosphopeptides from proteins that contribute to key CRC-associated oncogenic pathways, including the MAP-kinase, interleukin and p53 signalling pathways. TILs were found that responded to approximately a third of the phosphopeptides identified from the same tumour, producing IL-2, IFNg and TNFa. These TILs also exhibit cytolytic activity. In addition, immune responses to a small proportion of these phosphopeptides are seen in healthy donors.

Conclusions: We have identified over 20 novel tumour-specific CRC-associated phosphopeptide antigens. A functional cytotoxic T cell response to these phosphopeptide antigens is present in tumours. These novel tumour-specific antigens represent key targets for future tumour immunotherapy strategies in CRC.

P1359

IL17-producing CD161$^+$ CCR6+ T cells are associated with graft-versus-host disease after allogeneic stem cell transplantation

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Purpose/Objective: The C-type lectin-like receptor CD161 is a well-established marker for human IL17-producing T cells, which have been implicated to contribute to the development of graft-versus-host disease (GVHD) after allogeneic stem cell transplantation (allo-SCT).

Materials and methods: In this study, we analyzed CD161$^+$ T cell recovery, their functional properties and association with GVHD occurrence in allo-SCT recipients.

Results: While CD161$^+$ CD4$^+$ T cells steadily recovered, CD161^{hi}CD8$^+$ T cell numbers declined during tapering of Cyclosporine A (CsA), which can be explained by their initial growth advantage over CD161$^{neg/low}$CD8$^+$ T cells due to ABCB1-mediated CsA efflux. Interestingly, occurrence of acute and chronic GVHD was significantly correlated with decreased levels of circulating CD161$^+$ CD4$^+$ as well as CD161^{hi}CD8$^+$ T cells. In addition, these subsets from transplanted patients secreted high levels of IFNγ and more IL17 than healthy controls. Moreover, we found that CCR6 co-expression by CD161$^+$ T cells mediated specific migration towards CCL20, which was expressed in GVHD biopsies. Finally, we demonstrated that CCR6$^+$ T cells indeed were present in these CCL20$^+$ GVHD-affected tissues.

Conclusions: In conclusion, we showed that functional CD161$^+$ CCR6$^+$ co-expressing T cells disappear from the circulation and home to GVHD-affected tissue sites. These findings support the hypothesis that CD161$^+$ T cells actively participate in the pathophysiology of GVHD.

P1360

Immunological targeting of deregulated signalling in lymphoma: targeting a phosphorylated antigen derived from CD19

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Purpose/Objective: The nature of the antigens targeted by effective immunotherapeutic strategies is largely undefined. Cancer genome and epigenetic studies have revealed the importance of signal transduction pathway deregulation in the pathogenesis of cancer. Therapeutic strategies targeting these have been highly successful in the clinic, providing a powerful argument for aligning immunotherapeutic approaches to target signal transduction. The discovery that phosphorylation of serine and threonine residues is preserved during MHC class-I and II antigen processing suggests that phosphopeptide antigens derived from cancer-related phosphoproteins could serve as immunological signatures of 'transformed self'.

Materials and methods: Memory CD4$^+$ and CD8$^+$ T cells, which recognise a phosphorylated antigen derived from the cytoplasmic tail of CD19 (pCD19), can be isolated from the peripheral blood of healthy donors and grown as a T cell line. ELISpot and cytotoxicity assays have been used to determine the *in vitro* specificity and efficacy of T cells from both healthy donors and 11 patients with Chronic Lymphocytic Leukaemia (CLL). We are currently undertaking immunohistochemistry with a phosphospecific antibody to determine the distribution of the pCD19 epitope in B cell malignancies.

Results: Incubation of circulating lymphocytes with leukaemia cell lines, HLA-matched primary CLL cells, and mitogenically activated B cells leads to proliferation and expansion of pCD19-specific T cells, yet incubation with normal, healthy B cells does not. These expanded T cells are effective killers of cell lines, primary CLL cells and mitogenically activated B cells. Moreover, when compared to healthy controls, patients with CLL display diminished pCD19 specific immunity and immunohistochemistry has revealed the presence of the pCD19 epitope in a number of B cell malignancies.

Conclusions: These data show, for the first time, the presence of T cells which exhibit cytotoxicity against primary B-CLL cells through recognition of a posttranslationally modified antigen. Healthy individuals exhibit robust pCD19-specific immunity and abrogation of this is associated with CLL. The data increase our understanding of mechanisms within the adaptive immune response that target cells with underlying deregulated signalling, and validate the potential of phosphopeptides as immunotherapeutic targets.

P1361

Immunoreactivity to food antigens and changes in peripheral blood white cells subsets in patients with non-Hodgkin lymphoma

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Purpose/Objective: The aim of research was to determine the intensity of humoral and cellular immunity to food antigens: phytohemagglutinin (PHA), cow's milk proteins (CMP) and gliadin in patients with non-Hodgkin lymphoma (NHL). To evaluate the possible relationship of enhanced immunity with changes in peripheral blood white cells subsets, the percentages of lymphocytes, monocytes and granulocytes were determined.

Materials and methods: This study involved 48 patients with NHL before oncological therapy. The control group consisted up to 50 healthy volunteers. Levels of IgM, IgG and IgA antibodies to food antigens were determined by ELISA test. MTT test was used for examination of *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) by food antigens. Flow cytometry was performed for analysis of white blood cells.

Results: Enhanced levels of anti-CMP and anti-PHA IgA antibodies were found in patients with NHL (14 out of 44 and 10 out of 44, respectively). Furthermore, in 8 patients who had higher levels of anti-PHA IgA antibodies, reduced ability of PHA to stimulate proliferation of PBMC was found. Higher frequency of NHL patients (10 out of 18) with increased ratio of percentage of granulocytes and percentage of lymphocytes (GLR) had enhanced humoral immunity to food antigens. Decreased percentage of lymphocytes was observed in 19 out of 41 patients. In 11 mentioned patients enhanced levels of immunoglobulins to food antigens were determined. Our preliminary *in vitro* analysis of human leukocytes in 30% autologous plasma showed decrease in the percentage of lymphocytes treated with tested food antigens in comparison to the percentage of lymphocytes in control sample and sample exposed to antigen and N-acetylcysteine, indicating that oxidative reaction mediated by neutrophils could be the basis of observed lymphocyte destruction.

Conclusions: Data from our study demonstrate high frequency of NHL patients with increased GLR and with elevated humoral

immunity. These data suggest the need to elucidate to what extent presence of enhanced immunoreactivity to food constituents contribute to immune disturbances found in NHL patients. It is important to clarify the molecular mechanisms responsible for decrease in the percentage of lymphocytes and changes in GLR, observed in examined patients.

P1362

Increased AMPK phosphorylation and enhanced effector function following low dose ionising radiation of T cells

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Purpose/Objective: Radiation therapy (RT) is a standard treatment in many haematological and solid cancers aiming to cause irreparable damage to tumour cell DNA. However, circulating or tumour-resident immune cells are also exposed to ionising radiation (IR) during RT but this effect is less well studied. We previously observed that T cell responses to recall antigens were enhanced by low dose IR of PBMC *in vitro* (Tabi *et al.* J Immunol 2010, 185:1330). Here we study the characteristics and mechanism of low dose IR-induced enhancement of T cell function.
Materials and methods: The cells used in these experiments were PBMC from healthy donors or prostate cancer patients, T cells purified from PBMC, and peptide-specific $CD8^+$ T cell lines. Irradiation was carried out using a ^{137}Cs-source (0.637 Gy/min). T cell function was assessed by measuring cytokine production or CFSE dilution following stimulation with antigenic peptides or CD3/CD28 antibody coated beads. T cell cytotoxicity was measured by standard ^{51}Cr assay. The effect of IR and/or T cell stimulation on T cell signalling was tested by a phosphoprotein array and confirmed using phosphoflow antibodies.
Results: Low or standard dose IR (0.6−2.4 Gy) enhanced both peptide- and CD3/CD28 bead-induced T cell proliferation and IFNγ production. It also decreased the activation threshold of T cells by 10-fold following peptide stimulation and broadened the resulting Th1 cytokine profile. Cytotoxic activity displayed by a tumour antigen-specific T cell line was also elevated in a radiation dose-dependent and antigen-specific manner. T cells remained 'primed' to give enhanced responses to stimulation even 24 h after IR. A physiological parallel was observed in prostate cancer patients who received 2.0−2.4 Gy localised (pelvic) radiation. CD3/CD28 cross linking of the PBMC resulted in significantly elevated IFNγ and TNFα production 24 h after RT compared to that before radiation. T cells irradiated *in vitro* released ATP and displayed significant AMPK (T172) phosphorylation. This effect may contribute to the enhanced T cell function by altering the metabolic state of the irradiated cells.
Conclusions: We report here for the first time a direct effect of low dose radiation on T cells resulting in a significant enhancement of T cell function. This effect maybe exploited clinically to boost T cell responses in immunotherapeutic settings.

P1363

Increased CD4+ and CD8+ T cell differentiation in old melanoma patients

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Purpose/Objective: Constant antigen exposure is thought to drive end-stage T cell differentiation. This has been shown to be the case during chronic viral infections such as CMV and is particularly pronounced with age. Tumour burden may have a similar effect on T cell differentiation, especially in melanoma which is known to be highly immunogenic and to induce accumulation of tumour-specific T cells without evidence of disease amelioration. This study therefore sought to assess global T cell differentiation in stage I-III melanoma patients when compared to age matched healthy donors, aged between 60 and 90 years.
Materials and methods: Flow cytometry was used to analyse the T cells' CD45RA/CD27 phenotypic profile, CMV-specific T cell responses were measured via intracellular cytokine expression and Melan-A specific T cells were measured using peptide loaded MHC class I dextramers.
Results: When compared to healthy controls, melanoma patients had an inverted CD4:CD8 ratio. The CD4+ T cell compartment was shown to have a significant reduction in naïve ($CD27^+$ CD45RA+) cells that was accompanied by an increase in the central memory ($CD27^+$ CD45RA-) fraction (both $P < 0.05$). Among the CD8+ T cells, no change in naïve cell numbers could be detected, but a decrease in central memory cells was observed, with a slight increase in effector memory cells ($CD27^-$ CD45RA-) and a significant increase in effector memory cells re-expressing CD45RA ($CD27^-$ CD45RA+; $P < P < 0.05$). No concomitant increase in Melan-A specific CD8+ T cells was observed among HLA-A2 positive patients and donors. However, these trends were CMV independent and correlated in part with disease stage, affirming them to be a true attribute of melanoma burden.
Conclusions: In summary, CD8+ T cells seem more driven towards end-stage differentiation in melanoma patients. We are currently investigating both the functional characteristics of these highly differentiated cells and the differentiation profiles of skin resident T cells of melanoma patients and healthy controls.

P1365

Inflammation-driven intratumor lymphoid tissue in colo-rectal cancer

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Purpose/Objective: Immune infiltration is a fundamental component of solid tumors, however its significance to human cancer is still controversial. In colo-rectal cancer, inflammatory mediators are undoubtedly involved in cancer initiation as well as progression, however, the analysis of T cell infiltration in large cohorts of tumor patients has indicated a clear role of T cells in the anti tumor immune response, resulting in more favourable prognosis. Our aim was to determine whether ectopic lymphoid tissue is present in colo-rectal cancer andits possible contribution to disease progression.
Materials and methods: CRC patient samples were analyzed by immunohistochemistry. Two models of CRC were used: a preclinical model of colitis-associated colon cancer (AOM/DSS model) and a genetic model of colon cancer not driven by an inflammatory reaction (APCMin/AOM model). In mice, lymphoid aggregates were analyzed by immunofluorescence on colon frozen sections and colon whole mounts.
Results: Here we show the presence of intratumor lymphoid tissue in colo-rectal cancer (CRC), organized in high compartmentalized follicle-like structures, comprising of B and T cells and a network of

CD21+ follicular dendritic cells. To address the impact of ectopic lymphoid aggregates on tumor development and progression, we have analyzed a murine model of colitis-associated cancer (AOM/DSS) and found an expansion of B cell follicles in the mucosa of mice developing intestinal neoplasia. The increase is likely to be driven by the chronic inflammatory reaction, as is not induced in a non inflammatory model of intestinal neoplasia (APCMin). In CRC patients, high density of lymphoid follicles correlates with better prognosis for colo-rectal cancer patients. The presence of intratumor lymphoid neogenesis occurs by formation of new vessels, including high endothelial venules and lymphatic vessels and correlates with increased T cell infiltration, suggesting an active contribution to T cell traffic to the tumor and the induction of an antitumor immune response.
Conclusions: Our results suggest that intratumor lymphoid tissue in CRC correlates with a more favorable patient clinical outcome, rising the hypothesis that it takes part to the anti tumor immune response, providing a suitable site for cell interaction, actively participating to immune cell recruitment and activation.

P1366
Inflammatory and regulatory T cells contribute to a unique immune microenvironment in tumour tissue of colorectal cancer patients

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Purpose/Objective: Colorectal cancer is one of the five leading causes of cancer mortality worldwide. The mechanisms of pathogen clearance, inflammation and regulation by T cells in the healthy bowel are also important in controlling tumour growth. The majority of studies analyzing T cells and their relationship to colorectal tumour growth have focused on individual T cell markers or gene clusters and thus the complexity of the T cell response contributing to the growth of the tumour is not clear.
Materials and methods: Using a novel analytical flow cytometric approach in concert with confocal microscopy, we have studied the T cells in colorectal cancer patients and have defined a unique T cell signature for colorectal tumour tissue.
Results: We have shown that the tumour has a lower frequency of effector T cells (CD69+), but a higher frequency of both regulatory (CD25hi Foxp3+) and inflammatory T cells (IL-17+) compared with associated non transformed bowel tissue. We have also identified minor populations of T cells expressing conventional markers of both inflammatory and regulatory T cells (CD4+IL-17+Foxp3+) in the tumour tissue. These cells may represent intermediate populations or they may dictate an inflammatory versus regulatory function in surrounding T cells. To study the functional relevance of these cell subsets, methods have been established to isolate functional T cell subsets from tumour tissue and non-transformed bowel tissue. Effector T cells, regulatory T cells and inflammatory T cells have been isolated from tumour tissue and non-transformed bowel tissue from colorectal cancer patients. Regulatory T cells isolated from non-transformed bowel tissue actively inhibit proliferation of T cells isolated from the same tissue.
Conclusions: Together, these data describe an immune microenvironment in colorectal cancer unique to the tumour tissue and distinct from the surrounding healthy bowel tissue, and this distinct environment is reflected by a gradient of T cells expressing markers of multiple T cell subsets.

P1368
Is there any autoimmune destruction of lymphocytes driven by the oxidative burst in melanoma patients?

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Purpose/Objective: Decreased antitumor cellular immunity, (especially decreased percentage of lymphocytes), is reported to be present in many malignant diseases, while development ofTh2 immunity in general wasreported to be connected with bedprognosis of disease. Quantitative analysis of the serum immunoglobulins revealed that the enhanced levels of especially specificIgA immunoglobulins to tumor associated antigen calreticulin are presentin sera of majority of patients with hepatocellular, pancreatic, colorectal, or breastcancer. Pleass *et al.* 2007 showed that the interaction of Fc fragments of IgA or IgG (engaged with their antigens) with correspondingFc receptors on neutrophilstriggers theoxidative burst. The main proposal of this work is that metabolites of this oxidative reaction could nonspecifically damage neighboring lymphocytes. The aim of this work was to determine is there an increase in the ratio of the percentage of granulocyte and percentage of lymphocytes (GLR), present inpatients with melanoma, andis theobserved decrease of lymphocytes driven by the oxidative burst in melanoma patients?.
Materials and methods: Up to 90 melanoma patients and 36 healthy controls were included in the study. Determination of the percentages of white blood cell subpopulations was performed using flow cytometry.
Results: Of the 44 out from 90 melanoma patients had the enhanced (>5.10) GLR. Our preliminary analysis performed *in vitro* on the mixture of leucocytes in 30% autologous plasma obtained fromone melanoma patientshowed decrease in the percentage of lymphocytestreated with melaninin comparison to the percentage of lymphocytes in control sample and sample exposed to melanin and N-acetylcystein, inboth experiments :2 and 24 h after the beginning of incubation.
Conclusions: Data from this analysis are along with recent data on the presence ofenhanced GLRin some patients with malignant diseases andindicates that oxidative reaction mediated by the reaction of neutrophils with antibodies bound to their antigen may be the basis of the observed lymphocyte decreasein patients with melanoma.

P1371
Local complement activation abrogates the tumor-endothelial barrier and mediates T cell homing and tumor immune attack

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Purpose/Objective: Cancer immune therapy does not only depend on the ability of the patients to mount a specific tumor immune response but also to the capability of the effector cells to infiltrate tumors through the endothelial barrier. In Ovarian Cancer this is mediated by the endothelin-B receptor (ET_BR) as we previously described. For this reason we investigated if the endothelial barrier was an innate and irreversible characteristic of certain tumors that allows them to resist immune attacks, or whether it could be overcome or reversed.
Materials and methods: CD3+ T cells harvested from W. T. C57BL/6, HPV16 E6-E7 DNA vaccinated mice were adoptively transferred in tumor bearing: 1. W. T. C57BL/6 $^{+/-}$ C5a Receptor inhibitor, 2. complement component C3 (C3KO) deficient mice, 3. Decay accelerating factor 1 deficient mice (DAF-KO), C5a receptor (C5aRKO)

deficient mice. E7 specific CD8+ T cells infiltration was correlated with Tumor growth and with complement activation on tumor endothelial surface.
Results: We demonstrated that an anti-tumor specific E7-CD8$^+$ T cells infiltration required *in vivo* a local endothelial complement activation by anaphylatoxin release. Moreover T cell-released Th1 cytokines *in vitro* induced an endothelial C3 expression that promoted T cell adhesion to endothelium up-regulatingboth ICAM-1 and VCAM-1 surface protein by complement activation (C3a and C5a anaphylatoxin release).
Conclusions: This data suggests that initial T cell recognition of cognate tumor antigen by a sufficient number of tumor-reactive T cells can lead, via local complement activation and release of anaphylatoxins, to the up regulation of endothelial adhesion molecules and facilitated homing of additional tumor-specific effector T cells to the tumors, thereby establishing a positive feed-back loop that tunes T cell infiltration.

P1372

Mesenchymal contribution to leukocyte entry, transit, and positioning within human melanoma tumor tissues

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Purpose/Objective: Tumors are complex tissues that, in addition to cancer cells, contain many different cellular components (e.g. fibroblasts, endothelial cells and various types of leukocytes) and other non-cellular components (e.g. secreted soluble factors, collagens and fibronectin), that are collectively called tumor stroma or microenvironment. In order to understand factors that regulate leukocyte entry, migration and position within human cancer, we performed a comprehensive characterization of cellular and non-cellular components of human melanoma tissue microenvironment.
Materials and methods: We used a panel of tumor antigens to delineate the intratumoral area, and mesenchymal (cells and matrix), endothelial, and leukocyte markers to define stromal components by multicolor confocal microscopy. To systematize our study we quantified and analyzed separately two tissue regions: (1) the intratumoral area and (2) the peritumoral stroma.
Results: We found that peritumoral stroma contained a profusion of inflammatory postcapillary vessels, as indicated by the endothelial expression of the multichemokie receptor DARC, heavily surrounded by CD90 perivascular mesenchymal cells (MC). In addition, we purified CD90+ peritumoral mesenchymal cells by using multicolor fluorescence-activated cell sorting to further characterize this population *in vitro*, finding a secretory CCL2 stable proinflammatory phenotype. We show a complex interplay among tumor and non-tumor tissue cells resulting in general CCL2 expression in human melanoma, but restricted expression of its receptor CCR2 to blood monocytes and sparse leukocytes around peritumoral inflammatory vessels. Interestingly, CD90 expression was lost from a distinct intratumoral stromal network composed of FAP+ MC, which is a marker of activated tumor stroma, and extracellular matrix (ECM) proteins such as collagen 1 (COL1) and fibronectin (FN). A great heterogeneity among melanoma tissue samples was detected with regard to this intratumoral stromal network, showing areas with minimal, partial and high stromal infiltration. We found a positive correlation between intratumoral stromal components and leukocytes.
Conclusions: All together our results show that a variety of mesenchymal cells regulate tumor leukocyte recruitment, migration, and final positioning of leukocytes in human melanoma tissues, i.e. CD90+ cells surrounding peritumoral stroma vessels that express CCL2 and recruit inflammatory CCR2+ monocytes from peripheral blood, whereas specific intratumoral FAP+ MC organize a stromal scaffold that contact-guide invasion and final positioning of leukocytes within the tumor cell nests.

P1373

Mesenchymal sterm cells (MSCs) in head neck squamous cell cancer (HNSCC)

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Purpose/Objective: It is well established that cancer is not simply a disease confined to transformed epithelial cells, but is deeply influenced by the stromal microenvironment. In this contest a major role is played by immune cells, and by cells of mesenchymal origin. One of the solid tumors in which a potential role of MSCs has been proposed, is HNSCC, that is the sixth leading cancer by incidence worldwide.

The aim of this study was to demonstrate MSCs' presence in HNSCC, to evaluate of their immunosuppressive properties *in vitro* and to correlate MSCs frequency tumors extent.
Materials and methods: Twelve HNSCC affected patients has been enrolled in the study. The frequency of epithelial-, endothelial-cells, leucocytes, MSCs and fibroblasts, was determined by flowcytometry on fresh single cell suspensions: of cells 12 tumors and 6 controls specimens.
Results: Cells of mesenchymal origin resulting significantly enriched in tumors ($P < 0.05$). Form 5 patients we derived homogeneous adherent cells sharing morphology, immunophenotype and, more importantly, *in vitro* differentation potential with bone marrow-MSCs. Interestingly tumor derived MSC (tumor-MSC) showed a significant inhibitory effect on T cell proliferation in *in-vitro* models, in a dose dependent manner; this activity was, in part, IDO dependent. Finally a positive and significant correlation was observed between tumor extent and the frequency of tumor-MSC.
Conclusions: In conclusion we demonstrated that MSCs are enriched in HNSCC and have *in vitro* immunosuppressive activity on CD4+ T cells.

P1374

Monocyte chemoattractant protein 1 (MCP-1/CCL2) is associated with thymus atrophy in a mouse model of acute myeloid leukemia

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Purpose/Objective: Research works performed in acute myelogenous leukemia (AML)-affected patients have revealed T-cell immunodeficiency characterized by peripheral T lymphocytes unable to interact with blasts, reduced thymic emigrants and oligoclonal restricted repertoires. These observations suggest a profound thymic dysregulation which remains difficult to study in patients.
Materials and methods: As experimental limitations (absence of thymus biopsies, involution with age) in AML-affected patients hamper the study of T-cell immunodeficiency which could be associated with thymus dysregulation, we decided to address this question using an AML-inducible mouse model. Female C57BL/6-Ly5.1 mice were challenged intraperitonealy (IP) with a highly lethal, syngeneic AML cell line, C1498, transduced to express the Zsgreen fluorescent protein.
Results: Using the C1498 mouse AML model, we demonstrated that leukemia development was associated with thymus atrophy defined by abnormal weight, reduced cellularity and, increased necrosis. In addition, we observed a dramatic loss of peripheral CD4$^+$ and CD8$^+$ T cell numbers although the frequencies of CD4$^+$FoxP3$^+$ regulatory and memory CD3$^+$ T cells were increased. Investigating the mechanisms leading to this atrophy, we found significant intrathymic

levels of CCL2 chemokine, which was also highly expressed by leukemic cells when cultured *ex vivo*. Mice treated with a blocking anti-CCL2 antibody after leukemia induction revealed a lower tumor burden, no more thymic atrophy, augmented anti-leukemic T-cell responses and a better survival compared to AML-bearing mice.
Conclusions: These findings have potent implications for future immunotherapeutic strategies. In this study, a novel link between CCL2 and T-cell immunodeficiency in AML is proposed.

P1376
Nasopharyngeal carcinoma derived exosomes promote natural regulatory T cells (Treg) immunosuppressive properties

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Purpose/Objective: Exosomes are nano-vesicules found in large quantities in biological fluids and tumors of patients with nasopharyngeal carcinoma (NPC). These tumor exosomes play an important role in tumor progression due to their immunosuppressive properties. In addition, it has been reported that the frequency and suppressor functions of $CD4^+$ $CD25^{high}CD127^{low}FoxP3^+$ regulatory T cells (Treg) are also higher in NPC patients than healthy donors. As interections between NPC-derived exosomes and Tregs remain unknown, we investigated their ability to induce, expand, activate and recruit human Tregs.
Materials and methods: Human Tregs were isolated from blood of healthy donnors and co-incubated with exosomes purified from (1) culture supernatents of NPC cell lines (C15 exosomes) or (2) the plasma of healthy donnors (HD exosomes).
Results: C15 exosomes significantly increased Treg expansion and generated Treg Tim-3^{low} insensitive to the suppression induced by exosomes. C15 exosomes also significantly increased the level of expression of $CD25^{high}$ and $FoxP3^{high}$ on Treg and lead to the conversion of $CD4^+$ $CD25^-$ T cells into $CD4^+$ $CD25^{high}$ Treg. Moreover, co-incubation of C15 exosomes induces over expression of the genes associated with Treg phenotype (L-selectin, ICAM-1, OX40), their suppressive activity (IL10, TGF-β1, TNF-α, Tbet, Granzyme B), and recruitment (CCR6). These results are correlated with significant increase in the suppressive activity of Tregs in the presence of C15 exosomes ($p \leq 0.001$). Finally, the C15 exosomes are able to facilitate the recruitment of Treg cells via the chemokine CCL20.
Conclusions: Our results give new insights about NPC-derived exosomes immunoregulatory properties. They induce Treg expansion, up-regulate Treg suppressor function, enhance Treg chemo-attraction, and promote the conversion of $CD4^+$ $CD25^-$ T cells into Treg. Interactions of NPC-derived exosomes with $CD4^+$ regulatory T cells represent a newly-defined mechanism that might be involved in regulating peripheral tolerance by tumor cells and supporting immune evasion of human NPC.

P1377
Notch controls generation and function of human effector CD8 T cells

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Purpose/Objective: Generation of effector CD8 T cells with cytotoxic capacities is crucial for control of tumor growth. Dendritic cells (DC) provide important signals to promote activation and differentiation of naïve CD8 T cells into effective cytotoxic T lymphocytes (CTLs). In mice, Notch is known to regulate T cell responses. Our aim is to address how Notch affects human CD8 T cell priming and effector function.
Materials and methods: Primary human monocyte-derived dendritic cells (moDC) and naïve CD8 T cells were obtained from HLA-$A2^+$ donors. These cells were screened for the presence of Notch ligands and receptors, respectively. In addition, activation and differentiation of MelanA (Mart1)-specific CD8 T cells from these donors was studied in the presence or absence of Notch inhibitors. Additionally, a Mart1-specific T cell clone was used to address the role of Notch in activation of established effector T cells.
Results: Human moDC express Notch ligands Jagged1 and Delta-like4 upon maturation, whereas naïve CD8 T cells express Notch1. Inhibition of Notch during activation of CD8 T cells with autologous Mart1 peptide-pulsed moDC reduced the expansion of Mart1-specific CD8 T cells. This was accompanied by a decreased frequency of IFN-g-, TNF-a- and Granzyme B-producing CD8 T cells after antigen-specific activation, indicating that also effector functions of CD8 T cells are controlled by Notch. Furthermore, DC-mediated activation induced Notch signalling in the Mart1 specific T cell clone, increased surface expression of Notch1 and Notch2 and enhanced IFN-g secretion. Inhibition of Notch resulted in significant decreases in both the frequency of activated CD8 effector T cells as well as the amount of IFN-g secreted, demonstrating that Notch also modulates established effector cells.
Conclusions: The Notch pathway plays an important role in the priming of human naïve CD8 T cells as well as the activation of effector T cells. Enhancing Notch signaling between DC and naïve CD8 T cells could therefore promote anti-tumor responses.

P1379
Particular functional status of NK cells in melananoma patients: effect of chemotherapy

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Purpose/Objective: There is a growing incidence of melanoma for several decades. Malignant melanoma has a high metastatic potential and is difficult to cure. At the metastatic stage, 15−20% of patients respond to conventional chiomiotherapies and despite the promising clinical responses to target therapies, there is still no efficient treatment for metastatic melanoma. Numerous arguments indicate that melanomas are immunogenic and that the immune system can control the tumor growth in certain patients. Immunotherapy is an alternative of choice for melanoma patients resistant to chemotherapy. Natural Killer (NK) cells are important innate immunity effectors and their role in solid tumors is actually evaluated. NK activation is regulated by a balance between activating receptors that detect stress related molecules on targets and HLA-I specific inhibitory receptors.
Materials and methods: We have studied the phenotype and function of blood NK cells from 32 donors and from 64 melanoma patients at different stages of the disease: 8 stage I-II, 15 stage III and 24 stage IV patients. In addition, 17 patients were analysed 1 month post-chemotherapy.
Results: We first showed that NK cells infiltrate primitive melanoma and that they display a peritumoral distribution. We showed that the NK/T ratio is increased and the $CD56^{bright}$ subset is decreased in patients. Patient NK cells exhibit a co-reduced expression of $NKp46^{dim}$/$NKG2A^{dim}$ that is associated with a reduced functionality (cytotoxicity and IFNg secretion towards K562). We show that

chemotherapy modulate the phenotype and function of NK cells. Purified NK cells from pre or post-chemotherapy patients display distinct functional status. Melanoma cells express NK ligands for NKG2D, NCR and DNAM-1 that are involved in the lysis by NK cells.
Conclusions: These results provide arguments and new clues to design NK cell based immunotherapeutic strategies for metastatic melanoma.

P1380

Positive immunosuppressive mechanisms correlate with adaptive immune reaction and survival

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Purpose/Objective: Several studies in colorectal cancer (CRC) patients indicate a relationship between tumour immune infiltrates and clinical outcome. Here we analyse the correlation between immune cell prevalence with markers of co-inhibition or immune suppression. Our study aims to address the feasibility of three potential hypothesis; (1) increased immunosuppression and co-inhibition receptors are associated with tumour progression, increased relapse and poor survival; (2) patients with a strong adaptive immune signature and therefore good prognosis show a negative correlation with immunosuppression; or (3) patients exhibit both a strong immune response which positively correlates with increased levels of immunosuppression and cytotoxicity.
Materials and methods: Gene expression patterns of a panel of immunosuppression markers in 109 CRC patients were investigated using quantitative real-time PCR. Primary CRC (n = 109) FFPE tissue was analysed immunohistochemically for various immune cells at the tumour centre (CT) and invasive margin (IM). Fisher Exact-Test was used to assess the relationship between immune cell density and CRC TNM stage. ClueGO-cytoscape networking was implemented, along with an advanced correlation matrix, to achieve a comprehensive analysis of gene expression networks.
Results: Interestingly, we observed a strong positive correlation between infiltrating adaptive immune cells and markers of immunosuppression. A significant increase in the prevalence of programmed cell death 1 (PDCD1), programmed cell death 1 ligand (PD-L1), B- and T-lymphocyte attenuator (BTLA), CD80, CD86, CTLA4, Foxp3, B7H4 and IDO, positively correlated with markers of T cell activation, cytotoxicity (GNLY, GZMA/B/K, PRF1) and Th1 polarisation (IFN-g, Tbx21, IRF1). Furthermore, increased immunosuppression was observed in patients with the highest density of infiltrating cytotoxic T cells, memory T cells and Th1 cells. Thus, these data support the third hypothesis, showing a positive correlation between adaptive immunity and immunosuppressive entities in human tumours. Strikingly, despite elevated levels of immunosuppression, patients with a high adaptive immune response retained a correlation with low risk of relapse and prolonged survival.
Conclusions: The local *in situ* presence of high immunosuppression does not impair the beneficial effect of adaptive immune response on survival.

P1382

Potential tumor promoting effect of CD137 on Hodgkin lymphoma

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Purpose/Objective: Hodgkin Lymphoma is a type of solid hematological malignancy which its principal malignant cells, Reed Sternberg (RS) cells, only accounting for a small fraction of the total tumor mass. Large numbers of inflammatory cells make up the major part of the tumor mass. It is now accepted that RS cells are most frequently derived from germinal center B cells, nonetheless, how RS cells survive in this inflammatory environment remains unknown. Here, we identified that CD137, a member of TNFR superfamily, is expressed by RS cells as a neoantigen. In addition, we further explored the beneficial effects of ectopic CD137 to RS cells.
Materials and methods: a. CD137 expression on primary Hodgkin lymphoma lesion was determined by IHC staining.

b. KM-H2, a RS cell line, was used as experimental model. CD137 on KM-H2 cells was neutralized by neutralizing antibodies or silenced by RNA interference, and then co-cultured with PBMC or T cells. IFN-g release was used as the indicator of T cells activation.

c. CD137 ligand (CD137L) expression on KM-H2 cells was determined by using flow cytometry after CD137 neutralization or silencing.
Results: RS cells in primary Hodgkin lymphoma tissue sections stained strongly for CD137. When CD137 on KM-H2 cells was silenced or neutralized, greater IFN-γ release was induced in PBMCs and T cells which were cocultured with the KM-H2 cells. This induction of IFN-γ could be abrogated by addition of CD137L neutralizing antibodies, demonstrating that it was indeed due to CD137L. We also observed that CD137L expression on KM-H2 was up-regulated following CD137 knock-down or neutralization.
Conclusions: It was shown previously that CD137 is a strong co-stimulator of T cells which shares a similar downstream mechanism as CD28. Hence, we propose that ectopic CD137 expression on RS cells down-regulates CD137L on RS cells, and subsequently assists RS cells in evading immune surveillance by reducing T cells co-stimulation.

P1383

PTEN/MAPKa pathway plays a key role in platelet-activating factor-induced tumor metastasis

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Purpose/Objective: Platelet-activating factor (PAF), a potent inflammatory mediator, can enhance tumor growth and metastasis. In this study, we investigated the role of PTEN (Phosphatase and tensin homolog deleted on chromosome10), a dual specificity phosphatase with both lipid and protein phosphatase activities, in a PAF-induced experimental pulmonary tumor metastasis model.
Materials and methods: Immunoprecipitation and PTEN phosphatase assay, PI3K activity assay, *In vitro* Gene Transfection, *In vivo* Gene Delivery, Lung Colonization Assay.
Results: PAF injection resulted in an increase in AKT phosphorylation and a decrease in PTEN activity. Systemic administration of an adenovirus carrying PTEN cDNA (Ad-PTEN), but not the control Ad-LacZ, not only reversed PAF-induced changes in phosphorylation of PTEN and AKT, but also attenuated PAF-induced pulmonary metas-

tasis of B16F10. However, PAF-induced pulmonary metastasis was not inhibited by the PI3K inhibitor, Wortmannin. PAF injection resulted in phosphorylation of all three mitogen-activated protein kinases (MAPKs) in the lungs, and PAF-induced metastasis of B16F10 was inhibited by pretreatment of MAPKs inhibitors. Ad-PTEN abrogated PAF-induced MAPKs phosphorylation *in vivo* as well as *in vitro*.
Conclusions: These data indicate that PAF decreases PTEN activity, which, in turn, leads to phosphorylation of MAPKs, and, consequently, enhances tumor metastasis.

P1384
Regulation of the oncoprotein cortactin during CCR7 mediated breast cancer cells migration

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Purpose/Objective: The G-protein-coupled-receptor CCR7 through the binding to its ligands, the chemokines CCL19 and CCL21, induces chemotactic and invasive responses of breast tumor cells *in vivo* and *in vitro* triggering the development of ganglionar and distant metastases. Although it is well known that CCR7 triggers various signalling pathways the molecular mechanisms underlying CCR7-mediated chemotaxis and metastasis in breast carcinomas are not well understood. It is acceptable that cytoskeletal proteins located in the cell cortex coupling membrane dynamics to cortical actin assembly have a crucial role in these processes. In this direction, the oncoprotein cortactin is an important node in the regulation of the actin network and a key signalling protein in many cellular processes. Nevertheless, many basic aspects of cortactin regulation are unknown and little is the knowledge of its role in the migration mediated by chemokine receptors.
Materials and methods: Our work has investigated several questions about how cortactin activity is regulated and participates in CCR7 signalling and our findings reveal that both molecules may contribute to breast cancer progression through promotion of structures and processes which are essential for metastases.
Results: With fluorescence microscopy we demonstrated that CCR7 lignads induce a temporary translocation of cortactin from cytoplasmic compartments to the cell cortex where it accumulates at thin flat membrane extensions containing actin filaments called lamellipodia. CCR7 ligands binding also promoted an increment of specialized actin and cortactin-rich protrusions focused on degrading extracellular matrix termed invadopodia. Biochemical approaches confirmed that cortactin is a novel downstream effector of CCR7 and suggest that ERK mediates different phosphorylation events responsible for cortactin regulation following CCR7 activation. In addition, chemotaxis assays using phospho-mutant forms of cortactin pointed out that these phosphorylation events are crucial for CCR7-mediated migration and invasion of breast cancer cells.
Conclusions: Our findings suggest a novel mechanism by which the CCR7-ERK-cortactin pathway mediates functional maturation of invadopodia and lamellipodia and breast cancer cell invasion bringing us closer to understanding the link between CCR7 and breast cancer.

P1385
Regulatory T cells (Tregs), but not Th17, are increased patients with gastric adenocarcinoma

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Purpose/Objective: Cells of the immune system, such as regulatory T cells (Tregs) and Th17 have been involved in the surveillance or progression of tumours. Thus, we wished to investigate the prevalence of these subpopulations in a group of patients with gastric adenocarcinoma.
Materials and methods: Enriched T-cell populations were achieved from blood or gastric tissue (tumor and tumor-free) samples from 21 patients (14 with intestinal type and 7 with diffuse type disease). As a control, blood samples from 23 healthy subjects were used. The following cell markers were assessed by flow cytometry: CD4, CD8, CD25, FOXP3 and IL-17.
Results: The $CD4^+$ CD25+ (regulatory) population was found increased in T cell populations obtained from tissue samples, when comparing tumor with tumor-free gastric tissue in patients ($9 \pm 5.7\%$ versus $4 \pm 3\%$; $P = 0.014$). Likewise, intestinal type tumor shows higher FoxP3 and $CD4^+$ CD25+FoxP3+ percentages ($7.3 \pm 4.6\%$ and $2.7 \pm 2.4\%$, respectively) when compared to tumor-free tissue ($4.5 \pm 4.8\%$ and $1.6 \pm 2.6\%$; $P = 0.036$ and $P = 0.028$, respectively). Finally, the FoxP3+/IL17+ ratio is higher in blood samples of patients (2.2 ± 2.1) than in control subjects (1.1 ± 1.6; $P = 0.025$). No significant differences were found for CD4+ or CD8+ IL-17-producing cells.
Conclusions: Treg cells ($CD4^+$ $CD25^+$) and not Th17, seem to play a key role in tumor progression, more so in the intestinal type of gastric cancer.

P1386
Role of the tumor suppressor ARF in the alternative activation of macrophages

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Purpose/Objective: ARF is among the most widely inactivated gene in human cancer. The tumor suppressor activity of ARF is in part a result of its ability to prevent the degradation of p53. However, numerous evidences indicate that ARF also exhibits p53-independent functions and interestingly place ARF as a general sensor of different types of cellular stress. We previously demonstrated that ARF deficiency severely impairs inflammatory response *in vitro* and *in vivo*, establishing a role for ARF in regulation of innate immune responses. Based on these data, we postulated that ARF could modulate polarization of macrophages.
Materials and methods: Peritoneal macrophages were obtained from WT and $ARF^{-/-}$ mice. Basal expression of M2 markers were determined by quantitative PCRs in resting macrophages. Reverse transcription (RT)-PCR, microarray assays and Western blot analysis were employed to investigate the effects of M1 (LPS) or M2 (IL-4) activation on WT and $ARF^{-/-}$ macrophages.
Results: In this study, we demonstrate that resting $ARF^{-/-}$ macrophages express high levels of Ym1, and Fizz-1, two typical markers of alternative activation of macrophages. Additionally, $ARF^{-/-}$ cells showed an impaired production of proinflammatory cytokines/

chemokines in response to LPS. Moreover, after stimulation with IL-4, an inducer of alternative activation, well established M2 markers such as Fizz-1, Ym1, and arginase-1 were higher in $ARF^{-/-}$ cells compared with WT macrophages. Additionally, the cytokine and chemokine profile of $ARF^{-/-}$ macrophages was associated with a M2 phenotype. Interestingly, various pro-angiogenic factors such as VEGF and MMP-9 were also increased, suggesting the potential pro-tumoral action of $ARF^{-/-}$ macrophages.

Conclusions: In summary, these results indicate that ARF can contribute to the polarization and functional plasticity of macrophages.

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P1387

Simultaneous co-expression of memory- and effector-related genes by individual human CD8 T cells depends on antigen specificity and differentiation

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Purpose/Objective: Phenotypic and functional T cell properties are usually analyzed at the level of defined cell populations. However, large differences between individual T cells may have important functional consequences. To answer this issue, we performed highly sensitive single-cell gene expression profiling, which allows the direct *ex vivo* characterization of individual virus- and tumor-specific T cells from healthy donors and melanoma patients.

Materials and methods: HLA-A*0201-positive patients with stage III/IV metastatic melanoma were included in a phase I clinical trial (LUD-00-018). Patients received monthly low-dose of the Melan-A^{MART-1}_{26-35} unmodified natural (EAAGIGILTV) or the analog A27L (ELAGIGILTV) peptides, mixed CPG and IFA. Individual effector memory CD28+ (EM28+) and EM28- tetramer-specific $CD8^{pos}$ T cells were sorted by flow cytometer. Following direct cell lysis and reverse transcription, the resulting cDNA was precipitated and globally amplified. Semi-quantitative PCR was used for gene expression and TCR BV repertoire analyses.

Results: We have previously shown that vaccination with the natural Melan-A peptide induced T cells with superior effector functions as compared to the analog peptide optimized for enhanced HLA binding. Here we found that natural peptide vaccination induced EM28+ T cells with frequent co-expression of both memory/homing-associated genes (*CD27*, *IL7R*, *EOMES*, *CXCR3* and *CCR5*) and effector-related genes (*IFNG*, *KLRD1*, *PRF1* and *GZMB*), comparable to protective EBV- and CMV-specific T cells. In contrast, memory/homing- and effector-associated genes were less frequently co-expressed after vaccination with the analog peptide.

Conclusions: These findings reveal a previously unknown level of gene expression diversity among vaccine- and virus-specific T cells with the simultaneous co-expression of multiple memory/homing- and effector-related genes by the same cell. Such broad functional gene expression signatures within antigen-specific T cells may be critical for mounting efficient responses to pathogens or tumors. In summary, direct *ex vivo* high-resolution molecular characterization of individual T cells provides key insights into the processes shaping the functional properties of tumor- and virus-specific T cells.

P1388

Targeted-Therapy attenuates local and systemic immune suppression in patients with Soft Tissue Sarcoma favoring the onset of adaptive tumor immunity

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Purpose/Objective: Targeted-Therapies are designed to directly inhibit the growth of cancer cells. However it is becoming clear that this class of drugs can also exert multi-faced immune-related effects potentially contributing to their clinical efficacy. For Soft Tissue Sarcomas (STS), Targeted-Therapy has evolved as a standard treatment option. Nevertheless, little is presently known about nature and features of the immune response in these neoplasms and their possible contribution to treatment efficacy. The aim of this study is to characterize circulating immunoregulatory cell subsets and tumor immune infiltrates in patients with STS, to assess the immunomodulating potential of targeted-therapies in this clinical setting. Results observed in the subgroup of Solitary Fibrous Tumor (SFT) are here reported.

Materials and methods: Patients with malignant SFT receiving sunitinib, were enrolled in the study. PBMC were collected prior and at different time points during treatment and analyzed by multiparametric flow cytometry for the frequency and functional status of T regulatory cells (Treg) and myeloid-derived suppressor cells (MDSC). Local tumor immunity was evaluated by extensive immunoistochemical characterization in tumor specimens surgically removed before or after drug treatments.

Results: Peripheral Treg (defined as $CD4^+$ CD25hiFoxp3+) and MDSC (detected as $CD14^+$ CD11b+HLADRlo/neg cells), present at higher frequency with respect to healthy donors (Tregs 3.8% ± 0.5 versus 1.2% ± 0.2; MDCS 12.7% ± 4.99 versus 3.4% ± 0.9) were downmodulated by treatment, starting from the second week. However, disease progression occurring during treatment induced a boost in MDSC, that regained or exceed the pretreatment level. Immunohistochemical analysis showed the presence of a strong intratumoral CD3+ lymphocyte infiltration in the post-treatment tumor lesions, undetectable in untreated samples. Myeloid cells expressing M1 and M2 markers (i.e. CD68, CD163, CD209 and CD208) were present both in naïve and in treated tumors with enrichment in the M1 population in post-therapy specimens.

Conclusions: These data strongly suggest that sunitinib, revert a tolerogenic microenvironment in malignant SFT by parallel-immune mediate mechanisms, thus potentially favoring the setting of an adaptive immune response.

P1389

Temporally designed treatment of melanoma cells by ATRA and polyI:C results in enhanced chemokine and IFNß secretion controlled differently by TLR3 and MDA5

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Purpose/Objective: In the last three decades the incidence of melanoma has increased worldwide and no effective treatment modalities have yet been developed. ATRA and polyI:C are strong inducers of TLR3 and MDA5 expression, andpolyI:C-induced TLR3 andMDA5

signaling specifically causes cell death in melanoma cells *in vitro*. We addressed the question whether ATRA pre-treatment could enhance the efficacy of polyI:C and if so, would ATRA have any additional effects on this process.

Materials and methods: WM35 and WM983A human melanoma cells were obtained from ATCC and cultured according to the recommended protocol. Human dendritic cells (DCs) and macrophages were differentiated from blood-derived monocytes. $CD1a^-$ and $CD1a^+$ DCs were sorted by FACS DiVa. Relative mRNA expressions were analyzed by real-time Q-PCR. Protein expressions were measured by Western blot, the levels of secreted chemokines/cytokines were detected by ELISA. Small interfering RNA (siRNA) was introduced by electroporation. Migration assays were performed in transwell chambers.

Results: We found that combined treatment of human melanoma cells with ATRA and pI:C strongly increased the expression of TLR3 and MDA5 in both WM35 and WM983A cells associated to significantly higher mRNA and secreted levels of IFNβ, CXCL1, CXCL8/IL-8, CXCL9, and CXCL10 than cells treated with either ATRA or polyI:C. Silencing of MDA5 by siRNA moderately affected IFNβ secretion, whereas TLR3 knock down interfered with both CXCL chemokine and IFNβ production. Furthermore, supernatants of ATRA+polyI:C-activated cultures increased the migration of both human monocyte-derived macrophages and $CD1a^+$ dendritic cells significantly as compared to the supernatants of cells treated with either ATRA or polyIC, and this effect occured in a TLR3 dependent manner.

Conclusions: In conclusion, consecutive treatment with ATRA and polyI:C results in strong, TLR3/MDA5 mediated chemokine and interferon responses in cultured human melanoma cells, which triggers a functional migratory response in professional antigen-presenting cells. This novel mode of concomitant activation may offer a more efficient treatment option for future melanoma therapy.

P1391

The first encounter between tumor cells and immune system cells

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Purpose/Objective: Regulatory T cells (Tregs) play a key role in the immune response to tumors. In cancer patients, a high number of Tregs is indicative of a poor prognosis as their abundance is inversely correlated with survival. In contrast, tipping the balance in favor of effector T cells (Teffs) has been associated to favorable prognosis. The suppressive function of Tregs is considered one of the main obstacles to successful immunotherapy.

We have previously shown that at the time of tumor emergence, activated memory Tregs (amTregs) are the first to be involved in the immune response (Darrasse-Jeze *et al.* JCI 2009). Prior to Teffs, amTregs mount a secondary memory response against self antigens expressed by the tumor that overpowers Teffs, suggesting that the activation status of Tregs and Teffs dictates the tumor outcome.

Here we characterized the first encounter between tumor cells, Tregs and Teffs.

Materials and methods: We used a combination of genetic tools, fluorescent markers and multiphoton intravital microscopy.

Results: We described the dynamics of immune system cells within the microenvironment of an emerging tumor. Furthermore, we analyzed different conditions aimed to perturb tumor growth in order to identify the spatio-temporal mechanisms and the cellular dynamics that determine tumor growth or tumor rejection.

Conclusions: A better understanding of the dynamic behavior of immune system cells during the very early stages of tumor emergence will allow to identify new strategies for immunotherapy.

P1392

The HIF-1alpha hypoxia response in tumor-infiltrating T lymphocytes induces functional CD137 (4-1BB) for immunotherapy

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Purpose/Objective: To study the effects of hypoxia in the tumor microenvironment, as sensed by the HIF-1α system, on the expression of CD137 on tumor-infiltrating lymphocytes (TILs). To take advantadge of CD137 being expressed selectively on TILs and check the efficacy of immunotherapy with agonist anti-CD137 mAb delivered intratumorally.

Materials and methods: Mice: OT-1, OT-2, CD45.1, $Rag^{-/-}$ and MMTVneuT, Hif-1α floxed-UBC-Cre-ERT2 mice. Cell lines: CT26, B16-OVA and MC38. *In vivo* tumor growth: $0.5x10^6$ tumor cells were injected subcutaneously. Tumor hypoxia was measured by positron emission tomography (PET) with the radiotracer fluorine-18-fluoromisonidazole (18^F-FMISO). Flow cytometry: FACSCantoII and FACSCalibur (BD Pharmingen) as indicated were used for cell acquisition and data analysis was carried out using FlowJo. Tissue immunofluorescence staining was performed on 10-μm thick cryosections. Monoclonal antibodies for *in vivo* experiments: agonistic mouse anti-CD137 (clone 2A) and blocking anti-B7-H1 (PD-L1) (clone 10B5).

Results: The tumor microenvironment of transplanted and spontaneous mouse tumors is profoundly deprived of oxygenation as confirmed by PET imaging. CD8 and CD4 tumor-infiltrating T lymphocytes (TILs) of transplanted colon carcinomas, melanomas and spontaneous breast adenocarcinomas are CD137 (4-1BB) positive, as opposed to their counterparts in tumor draining lymph nodes and spleen. Expression of CD137 on activated T lymphocytes is markedly enhanced by hypoxia and the prolyl-hydroxylase inhibitor DMOG. Importantly, hypoxia does not up-regulate CD137 in inducible HIF 1α knock-out T cells, and such HIF-1α-deficient T cells remain CD137-negative even when becoming TILs, in clear contrast to co-infiltrating and co-transferred HIF-1α-sufficient T lymphocytes. The fact that CD137 is selectively expressed on TILs was exploited to confine the effects of immunotherapy with agonist anti-CD137 monoclonal antibodies (mAb) to the tumor tissue. As a result, low-dose intratumoral injections avoid liver inflammation, achieve antitumor systemic effects and permit synergistic therapeutic effects with PD-L1/B7-H1 blockade.

Conclusions: CD137 (4-1BB) is an important molecular target to augment antitumor immunity. Hypoxia in the tumor microenvironment as sensed by the HIF-1α system increases expression of CD137 on tumor-infiltrating lymphocytes which thereby become selectively responsive to the immunotherapeutic effects of anti-CD137 agonist monoclonal antibodies as those used in ongoing clinical trials.

P1393

The identification of the Cancer Associated Fibroblasts and functional analyses for mechanisms of immune evasion in a rat chemical mammary carcinoma model

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Purpose/Objective: Seed-centric cancer models do not take into account the probable impact of the microenvironment, soil, that the tumor cells reside in. Fibroblasts are thought to be involved in a dynamic crosstalk with other cells of the tumor microenvironment. The

aim of this study is to characterize and effectively isolate cancer associated fibroblasts (CAFs) for further functional assays.
Materials and methods: N-Nitroso N-Methyl Urea (NMU) induced experimental mammary carcinogenesis model was utilized. Twenty-one days old female Sprague Dawley rats were injected once a week for 4 weeks with NMU. When the animals are about 3 months old, developed tumors were harvested surgically under sterile conditions for CAF isolation. Fibroblasts were isolated from breast tumor tissues using a protocol which utilizes collagenase and hyaluronidase. Enzymatically digested tissues were then cultured in fibroblast selective medium. The same protocol was also used to isolate normal tissue fibroblasts from healthy mammary tissues. These CAFs and healthy tissue fibroblasts were immunostained to show their differential expressions of surface markers such as α-Smooth Muscle Actin (αSMA) and Vimentin, in order to distinguish CAFs from their normal tissue counterparts. Probable DNA damage of peripheral blood cells due to the NMU injections were evaluated by Comet Assays at different time points. Coculture of CAFs with lymphocytes and Carboxyfluorescein succinimidyl ester (CFSE) proliferation assays were performed for functional analyses.
Results: The immunostainings clearly showed that CAFs had significantly higher levels of αSMA expression than normal fibroblasts. This finding of differential αSMA expression was shown to fade out with further passages of cultured CAFs. Histological examinations also revealed significant morphological differences between these cells. The Comet Assays showed that levels of DNA damage in peripheral blood cells of tumor bearing animals were similar to control levels after 2 months post injection. CFSE proliferation assays showed the immunosuppressive effects of CAFs on lymphocytes (see Fig. 1).

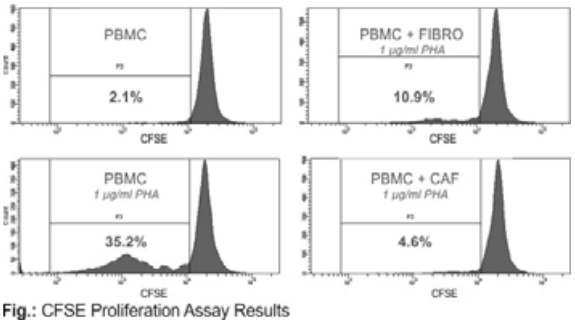

Fig.: CFSE Proliferation Assay Results

Conclusions: CAFs were successfully propagated using a rat chemical breast carcinogenesis model. Functional analyses with these CAFs were performed employing various coculture systems and showed their immunomodulatory roles on immunity against breast cancer.

P1394
The importance of epitope choice for adoptive T cell therapy

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Purpose/Objective: Due to high genetic instability large established tumors contain different cell variants, where some can be therapy resistant and lead to tumor escape. There are many factors contributing to the outgrowth of these escape variants that have to be taken into consideration for achieving optimal Results: . In this study we show the importance of epitope choice for a given target antigen by comparing rejection of large established SV40 Large T positive (T-Ag$^+$) tumors either by targeting subdominant epitope pI or immunodominant pIV.
Materials and methods: Retroviral transduction of mouse T cells, flow cytometry, cytokine release, MHC-peptide binding, T cell avidity, tumor challenge and adoptive T cell therapy, *in vivo* kill, *in vivo* bioluminescent imaging, western blot, proteasomal cleavage, mass spectrometry.
Results: T cells engineered to express pI or pIV specific T cell receptors (TCRs) were potent enough to long-term reject large established T-Ag$^+$ tumors. However, when tumors expressing low levels of MHC class I were treated, only TCR-I T cells rejected these tumors, while TCR-IV T cells selected IFNγ unresponsive escape variants. *Ex vivo* recognition of these escape variants was impaired for TCR-IV, but not for TCR-I T cells, indicating that in contrast to pI, pIV recognition was dependent on IFNγ stimulation. TCR-I and TCR-IV T cells had comparable avidity, and pI and pIV had similar affinity for the MHC class I molecule, therefore, the differential outcome of the two therapies could not be attributed to these factors. The difference was in the proteasomal cleavage of the two epitopes: pI was directly produced by the proteasome, while only N-terminal precursors were generated for pIV. Due to IFNγ unresponsiveness of the escape variants, ERAAP could not be upregulated by IFNγ stimulation.
Conclusions: We speculate that the N-terminal precursors could not be efficiently trimmed to generate the pIV epitope, allowing tumors to escape only TCR-IV but not TCR-I T cell therapy. We conclude that IFNγ independent epitopes might be better targets for adoptive T cell therapy of tumors expressing low levels of MHC class I.

P1395
The PD-1/PD-L1 axis contributes to T cell dysfunction in chronic lymphocytic leukemia

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Purpose/Objective: Chronic Lymphocytic Leukemia (CLL) is characterized by the progressive accumulation of a population of mature B lymphocytes. It is marked by profound defects in T-cell functions. Programmed death-1 (PD-1) is a cell surface molecule that inhibits activation and is involved in tumor escape mechanisms through binding of the specific PD-L1 ligand. The aim of this work is to evaluate expression and function of the PD-1/PD-L1 axis in CLL.
Materials and methods: We compared T cell subpopulations of CLL patients (n = 120) to age- and sex-matched healthy donors (HD, n = 30) using multiparameter flow cytometry. Immunohistochemical analyses were used to study PD-1 and PD-L1 expression in the lymph node microenvironment. Functional assays were used to determine the involvement of the PD-1/PD-L1 axis in shaping T cell responses.
Results: The first finding of this work is that CD4$^+$ T lymphocytes from CLL patients express significantly higher levels of the PD-1 receptor, as compared to the same cells purified from age- and sex-matched donors (52% versus 34%, $P < .001$). In keeping with the notion that PD-1 is a marker of cell exhaustion, we found that CD4$^+$ T lymphocytes from CLL patients display increased numbers of effector

memory cells with a concomitant decrease in naïve and central memory cells, when compared to age- and sex-matched donors. The number of effector memory cells positively associated with a more advanced stage of disease, treatment requirements and unfavorable genetic aberrations. Leukemic lymphocytes expressed higher levels of PD-L1 than circulating B lymphocytes from normal donors. PD-1 and PD-L1 increased significantly when T or B lymphocytes were induced to proliferate, suggesting that this interaction might work efficiently in an activated environment. This hypothesis was tested by determining PD-1 and PD-L1 expression in the proliferation centers located in the lymph nodes of CLL patients. Results indicate that PD-L1$^+$ proliferating CLL cells are in close contact with CD4$^+$/PD-1$^+$ T lymphocytes. Lastly, functional experiments performed using anti-PD-1 antibodies or recombinant PD-L1 ligands clearly indicate that this axis contributes to driving IL-4 secretion and to the inhibition of IFN-γ production by CD8$^+$ T cells.
Conclusions: Considered together, these observations imply that pharmacological manipulation of the PD-1/PD-L1 axis might be relevant in restoring T cell functions.

P1396
The phytochemical apigenin downregulates the inducible expression of programmed death ligand 1 (PDL-1) by mouse mammary carcinoma cells

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Purpose/Objective: PDL-1 is an inhibitory T cell molecule expressed by antigen presenting cells, activated T cells and various tumor types. PDL-1 expression by some breast cancer cell lines can be induced by the proinflammatory cytokine IFN-γ and is believed to contribute to immune evasion by breast cancer cells. Our goal is to determine the effect of the antiinflammatory dietary phytochemical apigenin on PDL-1 expression in a mouse model of breast cancer.
Materials and methods: The effect of apigenin on cell number and viability was determined using MTT assays and Annexin-V-FLUOS staining, respectively. The effect of apigenin on cell-surface PDL-1 was analyzed in mouse mammary carcinoma cell lines with IFN-γ-induced PDL-1 expression. Murine IFN-γ (1 ng/ml) was used to induce PDL-1 expression, which was measured by flow cytometry using PE-anti-human PDL-1 or isotype control antibodies.
Results: Apigenin at 10, 20 and 30 μM did not affect E0771 and 4T1 murine mammary carcinoma cell numbers by MTT assay; however, annexin V staining indicated a modest decrease in viability of both 4T1 and E0771 mammary carcinoma. IFN-γ-induced upregulation of PDL-1 on 4T1 and E0771 mammary carcinoma cells was significantly decreased by apigenin.
Conclusions: Apigenin and other phytochemicals that downregulate inducible PDL-1 expression may represent a new class of chemotherapeutics that will increase the vulnerability of breast cancer cells to antitumor immune responses.

P1397
The potential role of eosinophils in the microenvironment niche of Multiple Myeloma

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Purpose/Objective: Multiple myeloma (MM) is a B cell neoplasm characterised by aberrant expansion of plasma cells within the bone marrow. It is thought that survival of these neoplastic plasma cells, similar to 'normal' plasma cells require survival signals from their environment. Recently, we and others have shown that in addition to bone marrow stromal cells, hematopoietic cell types such as megakaryocytes and eosinophils also play an important role in the function of plasma cell niches. Here, we tested the hypothesis that MM cell growth is also supported by these components of plasma cell niches.
Materials and methods: A novel MM mouse model, i.e. a subclone of the BALB/c derived plasmacytoma cell line MOPC 315 that specifically homes to bone marrow of recipient mice, which lead to symptoms closely resembling the human MM disease was used in these studies. Eosinophils were depleted from wild type BALB/c mice by injection of the anti-IL5 blocking antibody. Serum were taken from mice every week and myeloma load was quantified by measuring MOPC specific dnp antibody titres by ELISA.
Results: Anti-IL5 treatment lead to approximately 30–50% reduction of bone marrow eosinophil counts for at least 4 weeks. There was a significant difference in dnp antibody titres after 2 weeks however this was not seen in the subsequent 2 weeks and there was no difference in the percentage of MOPC 315 after mice were sacrificed at 5 weeks.
Conclusions: This study shows that their could be a supportive role for eosinophils in the earlier stages of the disease, but not necessarily in the later stages. It would be interesting to test this further in a eosinophil depeleted mice to see if the changes seen is more drastic.

P1399

The telomeric protein TRF2 restrains a cell-extrinsic pathway by which cancer cells are eliminated by natural killer (NK) cells

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Purpose/Objective: Cells respond intrinsically to the perception of DNA damage by initiating the DNA damage response (DDR) pathway that leads to cell-cycle arrest, senescence or apoptosis. Telomeres protect chromosome extremities from being repaired and recognized as accidental double strand DNA breaks. For instance, loss of TRF2, a major telomere protein in mammals, triggers an ATM-dependent DDR, telomere fusion and growth arrest. As we observed during early stages of human colon cancer the progressive TRF2 upregulation correlated with a decrease in NK cells density, we address the question of a possible link between TRF2 dosage and cancer immunity.

Materials and methods: Two cellular models are used: (1) immortalized human fibroblast overexpressing or not the oncogenic form of Ras or (2) the mouse melanoma B16 cells. Using lentivirus, we modulated TRF2 expression (overexpression or knockdown), and we analyzed cellular effects of TRF2 modulation *in vitro*. Tumorigenicity of transduced cells was analyzed both in immunocompromised and immunocompetent mice. Immune microenvironment was analyzed 5 days after tumor engraftment. Finally, NK cells functionality was assayed by CD107a degranulation assay.

Results: In a Ras V12 contexture, we observed that TRF2 modulation do not impact neither tumor cell proliferation nor clonogenicity *in vitro and inhibit DDR pathway activation: not clear, I thought that TRF2 downregulation does not activate DDR*. We observed that TRF2 overexpression in both human transformed cells and mouse melanoma cells decreases their ability to activate NK cells *in vitro*. Conversely, a reduced TRF2 dosage can impair tumorigenicity without engaging a cell intrinsic program of proliferation arrest but by an ATM-independent mechanism activating natural killer (NK) cells in both models. In addition, we show that TRF2 dosage modulates NK cell infiltration within the tumor microenvironment and global modification of immune infiltrate.

Conclusions: We demonstrate here that TRF2 dosage in cancer cells modulates their ability to activate NK cels. We propose that the increased level of TRF2 measured in many human tumors can favor cancer progression by a cell extrinsic mechanism contributing to bypass immunosurveillance.

P1401

Tumor-infiltrating regulatory T cells favor tumor development by suppressing anti-tumor immunity in patients with primary and metastatic liver cancer

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Purpose/Objective: The mechanisms that enable liver cancer to escape elimination by the immune system remain unclear, but their elucidation may provide novel therapeutic interventions.

Materials and methods: We investigated the impact of tumor-infiltrating regulatory T cells on tumor-specific T cell responses in patients with liver cancer, using *ex vivo* isolated cells from individuals with hepatocellular carcinoma (HCC) or liver metastases from colorectal cancer (LM-CRC).

Results: We observed the presence of tumor antigen specific responder CD4+ T cells in the blood of these patients, but they were undetectable in tumor infiltrating lymphocytes (TIL's). In both HCC and LM-CRC, $CD4^+$ CD25+Foxp3+ regulatory T cells (Treg) accumulate in the tumor milieu and are potent suppressors of autologous tumor-specific T cell responses. We show that tumor Treg up-regulate the expression of glucocorticoid-induced tumor necrosis factor receptor (GITR) and the inducible T-cell co-stimulator (ICOS) compared to Treg in tumor-free liver tissue and blood. Importantly, treatment with soluble GITR ligand (sGITRL) induces a decrease in the suppression mediated by the activated tumor-infiltrating Treg and restores the proliferative capacity and cytokine production of $CD4^+$ CD25- T cells.

Conclusions: Our results show that tumor-associated Tregs are critical for immune evasion in liver cancer and we propose that GITRL constitutes a rational treatment for this disease.

P1402

Tumor-specific CD4+ T cells develop cytotoxic activity and eliminate virus-induced tumour cells in the absence of regulatory T cells

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Purpose/Objective: The majorities of tumor viruses is well controlled by the immune system and therefore cause only transient or no disease in their hosts after infection. The important role of tumour-specific cytotoxic $CD8^+$ T cells (CTLs) is well-defined in the immune control of the tumours but the role of effector $CD4^+$ T cells is poorly understood. In addition, the significance of regulatory T cells (Tregs) in inhibiting tumor-specific $CD4^+$ T cell responses during tumor rejection *in vivo* has not been defined.

Materials and methods: In the current research we have used a murine retrovirus-induced tumour cell line of C57BL/6 mouse origin, namely FBL-3 cells, as a model to study basic mechanisms of immunological control and escape during tumour formation. In the present study we used Foxp3 transgenic mice (DEREG) expressing the diphtheria toxin receptor under the control of the Foxp3 promoter, which made it possible to selectively deplete Tregs *in vivo* and to determine the influence of $Foxp3^+$ Tregs on T cell responses during tumor regression. FBL-3 tumor cells were injected subcutaneously on the right flank of the mouse. Tumor size based on caliper measurements was calculated. At different time points mice were sacrificed, and

tumors and draining and non-draining lymph nodes were resected. Data were acquired on an LSRII flow cytometer. Analyses were done using FACSDiva software (Becton Dickinson) and FlowJo software (Treestar).

Results: This study shows that tumour-specific $CD4^+$ T cells are able to protect against virus-induced tumour cells. We show here that there is an expansion of tumour-specific $CD4^+$ T cells producing cytokines (IFN-γ, TNF-α and IL-2) and cytotoxic molecule granzyme B in the early phase of tumour growth. Importantly, we demonstrate that *in vivo* depletion of Tregs and $CD8^+$ T cells in FBL-3-bearing DEREG transgenic mice augments IL-2 and granzyme B production by $CD4^+$ T cells and increases FV-specific $CD4^+$ T-cell effector and cytotoxic responses leading to the complete tumour regression.

Conclusions: Therefore, the capacity to reject tumour acquired by tumour-reactive $CD4^+$ T cells largely depends on the direct suppressive activity of regulatory T cells. We suggest that a cytotoxic $CD4^+$ T cell immune response may be induced to enhance resistance against oncovirus-associated tumours.

P1403

Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and Treg cells

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Purpose/Objective: Although immune mechanisms can suppress tumor growth, tumors establish potent and overlapping mechanisms that mediate immune evasion. Emerging evidence suggests a link between tumor immune tolerance and angiogenesis. Hypoxia, a condition known to drive tumor angiogenesis, results in release of damage-associated molecular pattern molecules, which can trigger tumor immune rejection. Thus, counter-activation of tolerance mechanisms at the site of tumor hypoxia would be a critical condition to maintain immunologic escape of tumors. Direct link between tumor hypoxia and tolerance through the recruitment of regulatory cells has not been established.

Materials and methods: We used early-passage primary cell lines from 4 solid ovarian cancers and 3 ascites and 10 established human ovarian cancer cell lines. ID8-ccl28 cells were derived from ID8 cells through transfection of codon-optimized *ccl28* cDNA cloned into pCDNA3 vector. For hypoxia experiments, cells were cultured for 16 h under hypoxic (1.5% O_2) or oxic conditions (21% O_2) and 5% CO_2 at 37°C. We used 6–8 week old female C57BL/6 mice to establish intraperitoneal ID8 or ID8-ccl28 tumours. *In vivo* depletion of $CD4^+$ $CD25^+$ cells was achieved with i.p. anti-CD25 Ab (PC61) or immunotoxin developed with anti-mouse CCR10 or CCR3 Ab, conjugated at equimolar ratio with Strep-ZAP. Two publicly available Affymetrix expression array datasets, comprising a total of 353 human ovarian cancer patients, were mined to analyze the interaction of *CCL28* with survival.

Results: Tumor hypoxic media recruited significantly more $CD4^+$ $CD25^+FoxP3^+$ cells relative to oxic media. The ability of hypoxic media to recruit preferentially $CD4^+$ $CD25^+FoxP3^+$ cells was abrogated by antibody neutralizing human CCL28. ID8-ccl28 tumors accumulated significantly more $CD4^+$ $CD25^+FoxP3^+$ cells *in vivo* relative to ID8 tumors. OrthotopicID8-ccl28 tumors exhibited significantly faster growth and ascites development relative to ID8 tumors. Depletion of CCR10 positive cells suppressed tumor growth and abrogated the effects of ccl28 overexpression.

Conclusions: Here we show that tumor hypoxia promotes T regulatory (Treg) cell recruitment via the CCL28 chemokine, which in turn promotes tumor tolerance and angiogenesis. Thus, peripheral immune tolerance and angiogenesis are intimately connected and cooperate to sustain tumor growth.

P1404

Tumour-Associated MDSC in Malignant Pleural Mesothelioma

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Purpose/Objective: We previously identified 5T4, a 72 kDa oncofetal antigen, on all malignant pleural mesothelioma (MPM) cell lines, pleural fluid (PF)-localised tumour cells and tumour tissue tested by flow cytometry, immunohistochemistry and Western blotting ($n = 42$). We observed $CD8^+$ and $CD4^+$ 5T4-specific T cell responses in the peripheral blood, but to a lesser extent in the tumour associated PF of patients. This indicated that localised immunosuppression was possibly hindering immune interactions. The relevance of regulatory T cells (Tregs) in MPM is not proven, whilst the presence of suppressive monocytes, such as myeloid derived suppressor cells (MDSC) has not been previously addressed. We aimed to elucidate the presence and relevance of Tregs and MDSC in patients as they may need to be considered for optimal immunotherapy.

Materials and methods: Flow cytometry was used to detect Tregs ($CD4^+$ CD25hi$Foxp3^+$) or MDSC (defined here as $CD3^-$ $CD14^-$ HLA-DR^- cells located within the monocyte gate). Mixed lymphocyte reaction (MLR) was carried out by stimulating allogeneic T cells with either $CD14^+$HLA-DR^+ classical monocytes or with $CD3^-$ $CD14^-$ HLA-DR^- cells. T cell proliferation was measured with a thymidine incorporation assay. Antigen-specific T cell stimulation was conducted over 6 days using PF cells pulsed with viral, 5T4 or common tumour antigen peptide pools, followed by intracellular IL-2/IFN-γ flow cytometry staining.

Results: We found that the levels of Tregs in MPM patients were not significantly elevated above those found in healthy blood. However, we identified a $CD14^-$ HLA-DR^- subset of cells with highly upregulated CD11b, CD15 and CD33 in the PF, when compared to the same population in patient and healthy blood. These cells also showed upregulation of the suppressive marker CD200 and were shown to suppress T cell proliferation in a MLR. Furthermore, they were able to suppress T cell IL-2/IFN-γ production in response to antigenic stimulation in some patients.

Conclusions: We report here for the first time the presence of MDSC in MPM patients. These MDSC are functional in patient PF, significantly decreasing T-cell proliferation and cytokine production in response to both antigen-specific and non-specific stimulation. MDSC may contribute to tumour-related immunosuppression in the tumour environment in MPM.

P1405

Use of Telomerase-derived Universal Cancer Peptides for dynamic monitoring of CD_4 T cell responses and cancer vaccines improvement

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Purpose/Objective: The stimulation of CD4+ T helper cell responses has gained considerable interest for cancer immunotherapy. We evaluate the antitumor potential of CD4 T cells specific of novel telo-

merase-derived peptides referred as universal cancer peptide (UCP) that bind to most commonly found HLA-DR alleles.
Materials and methods: We prospectively studied a cohort of 84 advanced NSCLC patients for the presence of naturally occurring UCP-specific CD4 T cells prior chemotherapy (CT). A relevant preclinical HLA-A2/DR1 transgenic mouse model was used to evaluate the potential of UCP-based cancer vaccine.
Results: Significant frequency (38%) of naturally occurring UCP-specific CD4 T-cell responses were detected before CT in advanced NSCLC but not in healthy volunteers. UCP-specific CD4 T-cell clones generated from cancer patients exhibited high avidity and are Th1 polarized. Interestingly, the presence of UCP-specific T cell response was shown to significantly increase overall survival (OS) of patients responding to CT (Median OS: 53 versus 40 weeks, $P < 0.034$). By using a HLA-A2/DR1 transgenic mouse model, we showed that UCP-specific CD4+ T cells induced after vaccination fulfilled helper features necessary to generate potent tumor-specific CTL responses. Furthermore the use of UCPs in therapeutic vaccination breaks self tolerance against telomerase and eradicates established mouse melanoma by promoting massive CD8+ T cells recruitment at the tumor site.
Conclusions: Together with the presence of natural UCP-specific T cell responses in many other cancers, these results support that the stimulation of UCP-specific CD4+ helper T cells is a powerful method to improve cancer vaccines efficiency and provides a new tool for comprehensive monitoring of antitumor responses in cancer patients.

P1406
VEGF-A-induced Treg proliferation, a novel mechanism of tumor immune escape in colorectal cancer: effects of anti-VEGF/VEGFR therapies

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Purpose/Objective: Regulatory T cells (Treg) are suspected of hindering an effective antitumor immune response in cancer. Multi-target anti-angiogenic tyrosine kinase inhibitors (TKI) that are routinely used as first or second line treatment of cancer patients, have been shown to decrease Treg proportion in tumor-bearing mice and metastatic renal cancer patients. However, the role of VEGF/VEGFR blockade in this effect is still debatable, and the direct impact of VEGF-A on Treg has not been studied.
Materials and methods: Treg proportion, number were analyzed by flow cytometry in peripheral blood of metastatic colorectal cancer (mCRC) patients treated with bevacizumab, and in CT26 tumor-bearing mice treated with drugs targeting the VEGF axis. The direct impact of VEGF on Treg increase in cancer was also studied.
Results: Sunitinib (a TKI targeting VEGFR, PDGFR, c-kit), and anti-VEGF-A antibody both decreased Treg in CT26 tumor-bearing mice. Masitinib, a TKI that does not target VEGFR, did not reduce Treg proportion in CT26 bearing mice. Bevacizumab, an anti-VEGF-A monoclonal antibody, reduced Treg proportion in peripheral blood of mCRC patients. Proliferation of Treg was enhanced in CT26 tumor-bearing mice compared to tumor-free mice and was decreased after anti-VEGF-A treatment. Furthermore, *in vitro* experiments have shown that VEGF-A could directly induce Treg proliferation. VEGFR1 and 2 were expressed on Treg in the presence of a tumor. Anti-VEGFR2 antibody administration reduces Treg proportion and also proliferation in CT26 bearing mice, but anti-VEGFR1 antibody did not, suggesting that VEGF-A-induced Treg proliferation was dependent on VEGFR2 expression. In metastatic CRC patients, Treg proliferation was also enhanced compared to healthy volunteers and was blocked by bevacizumab treatment.
Conclusions: We identified a new mechanism by which VEGF-A induced by the tumor could stimulate Treg proliferation. VEGF-A/VEGFR2 blockade reduced Treg proportion and proliferation in tumor-bearing mice and metastatic CRC patients suggesting that combination of anti-VEGF-A/VEGFR2 therapies with immunotherapeutic approaches in the future might be particularly relevant in CRC patients.

P1407
IFN inducible genes, programmed cell death and cancer

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Purpose/Objective: The Interferon inducible transmembrane protein family (IFITM) consists of several members, highly homologous and conserved among species. The IFITM genes are involved in multiple cellular processes: early development, immune response, inflammation, malignant transformation, cell cycle control etc. They were recently found to powerfully inhibit virus infection *in vitro* and to restrict the morbidity and mortality induced by Influenza infection *in vivo*. Members of the IFITM family are overexpressed in inflamed and malignant tissues, in mice and humans. Moreover, IFITM expression was shown to be altered during malignant transformation in the gastro-intestinal tract. We have highlighted the IFITM proteins as putative immunodominant tumor-associated antigens of colon carcinoma, since peptides derived from the IFITM2 protein induced both immunogenic and antigenic responses in humanized mice. We have previously reported that exogenous overexpression of IFITM2, induced apoptosis in various cell lines, through the intrinsic mitochondrial pathway, independently of p53. Furthermore, we have shown that overexpression of wild-type p53 (WTp53), downregulated IFITM2 steady-state protein levels.
Materials and Methods: As a part of our effort to characterize the IFITM2 gene, its functions and contribution to tumorigenesis, the presented study was designed to establish the detected downregulation in IFITM2 expression levels by WTp53 and to investigate the mechanism of suppression.
Results: Here we show that the regulation of endogenous IFITM2, at the protein level, is specifically p53-dependent and cell-independent. On the other hand, the regulation of IFITM2, at the RNA level, is cell and condition-specific. We demonstrate that the action of p53 on IFITM2 levels requires the integrity of the DNA binding and transactivation domains. Thus, it is probably mediated by a DNA-bound form of p53. This indicates on participation of another, yet unidentified, factor in this regulation. Finally, we show that IFITM2 is degraded by the lysosomal and not by the proteosomal pathway.
Conclusions: Further investigation of the precise mechanisms, by which IFITM2 is regulated, as well as its functional activity in tumor microenvironment are of great importance in attributing to our understanding of the cancer pathology.

Poster session: Veterinary Immunology

P1408

A sandwich ELISA for quantitative detection of transcription factor of porcine natural regulatory T cell

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Purpose/Objective: The transcription factor forkhead box P3 (Foxp3) is not only a master regulator for the development and the function of natural regulatory T cell (nTreg), but also the most specific marker identified thus far for nTreg. To understand the role of Foxp3 in all sorts of immune responses in swine, it is necessary to determine the function and the expression level of Foxp3. A sensitive and specific indirect sandwich enzyme-linked immunosorbent assay (ELISA) for detecting porcine Foxp3 was developed.
Materials and methods: The prokaryotic expression vector pET-28a-Foxp3 transformed into host strain *E. coli* BL21. The fusion protein about 48 kDa was induced by IPTG and purified with Ni-NTA Agarose. The rabbit antiserum against porcine Foxp3 protein was obtained by immunizing a rabbit with purified fusion protein. In addition, five hybridoma cell clones (2A4, 2C4, 3F3, 4C8 and 4C11) were obtained, which can be cultured stably and secret McAb against porcine Foxp3 protein. By Protein G affinity chromatography, McAb 4C8 and the antiserum were purified and then used as coating antibody and detecting antibody, respectively.
Results: The relationship between ELISA signal and concentration of Foxp3 was linear with an R^2 of 0.9981. Minimum detectable concentration was 0.458 ng/ml. Using this ELISA, samples of 17 diseased pigs and 27 disease-free pigs were detected. Mean and standard deviation of the above two groups were 108.82, 85.77 and 167.54, 89.97, respectively. One-way ANOVA analysis showed that the difference was significant ($P < 0.05$).
Conclusions: An indirect sandwich ELISA for detection of porcine Foxp3 with McAb and PcAb was established and it will be useful to detect porcine Foxp3 and nTreg.

P1409

Adenovirus-mediated canine interferon-γ expression and its antiviral activity to canine parvovirus

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Purpose/Objective: To investigate adenovirus-mediated IFN-γ gene transductionand its antiviral activity to CPV in canine kidney cells (MDCK).
Materials and methods: The cIFN-γ gene amplified by RT-PCR was inserted into adenovirus shuttle plasmid to construct pShuttle3-cIFN-γ vector, from which the cIFN-γ expression cassette was transfered into the adenovirus genomic plasmid pAdeno-X by specific restriction sites to generate recombinant adenovirus genomic plasmid pAd-cIFN-γ. The plasmid pAd-cIFN-γ was linearized by Pac Idigestion and transfected into human embryonic kidney (HEK) 293T cells to generate the replication-defective cIFN-γ recombinant adenovirus. The cIFN-γ recombinant adenovirus was identified by PCR and the recombinant cIFN-γ expression was identified by Western blot. To analyze its anti-canine parvovirus activity, the MDCK cells were pre-infected by cIFN-γ recombinant adenovirus, and then infected by canine parvovirus. The antiviral activity of the cIFN-γ recombinant adenovirus against parvovirus was evaluated based on the parvovirus titer reduction.
Results: The results showed that the recombinant adenovirus containing cIFN-γ gene was successfully constructed by the ligation method. Western-blot analysis showed the recombinant adenovirus could mediate cIFN-γ secretory expression in MDCK cells. The cIFN-γ recombinant adenovirus could significantly inhibit canine parvovirus replication in MDCK cells pre-infected with the recombinant virus.
Conclusions: The cIFN-γ recombinant adenovirus has the potent antiviral activity to canine parvovirus.

P1410

Anti-canine parvovirus activity of recombinant canine soluble transferring receptor prepared with codon-optimized gene construct

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Purpose/Objective: To inhibit canine parvovirus (CPV) infection using recombinant soluble canine transferrin receptor (sTfR), the receptor of CPV.
Materials and methods: The codon-optimized sTfR gene was inserted into pcDNA3.1A-CD5 plasmid by fusing His-tag to the C-terminus and human CD5 signal peptide to the N-terminus of sTfR, respectively. The sTfR vector was then transfected into HEK 293FT cells, the expressed sTfR was purified from culture supernatants by Ni-NTA agarose affinity chromatography and identified by Western-blot. Furthermore, the binding ability of sTfR to CPV and CPV envelope protein 2 (VP2) were analyzed by ELISA., and the sTfR antiviral activity against CPV was evaluated by viral titer reduction in feline F81 cells.
Results: The results showed that codon optimization significantly enhanced (a more than twofold increase) sTfR expression compared with wild-type sTfR. Moreover, the recombinant sTfR is able to specifically bind to CPV and VP2 proteins and also capable of preventing CPV infection in the host cells.
Conclusions: The recombinant sTfRpossesses the antiviral activity to CPV, which provides the solid foundation for further investigation of sTfR anti-CPV activity with dogs *in vivo*.

P1411

CCL2 and CCL5 induce LPS-insensitive S100A8/A9-positive bovine macrophages

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Purpose/Objective: Little is known about macrophage plasticity in the bovine system, where two mutually exclusive subpopulations can be identified in tissues. The objective was a) to analyze whether monocyte-attracting chemokines CCL2 and CCL5 skew the differentiation of macrophages into CD163+ or S100A8/A9+ cells and b) to analyze the functional phenotype of CCL2/CCL5-differentiated macrophages.
Materials and methods: MACS-separated peripheral blood monocytes (using a mouse anti-human CD172a mAb) were phenotyped for their CD14 expression. RboCCL2 or rboCCL5-induced $Ca2^+$ influx was assessed flow cytometrically with Fluo-4-loaded monocytes. Monocytes were cultivated *in vitro* for up to 7 days in the presence or absence of rboCCL2, rboCCL5. At day 4 and day 7 cells were analyzed flow cytometrically for their expression of CD163 and

S100A8/A9. Day 7 macrophages were stimulated for 3 h with LPS. Quantitative RT-PCR was performed for CXCL1, CXCL8, Arginase, iNOS, IL-10, and IL-12.

Results: Bovine monocyte subpopulations were identified as $CD172a^{+}/CD14^{high}$ (71.8%) and $CD172a^{+}/CD14^{low}$ monocytes (28.4%). CCL5-mediated Ca^{2+} influx in monocytes was significantly stronger than CCL2-mediated cell activation (with $CD14^{high}$ monocytes responding more sensitive than $CD14^{low}$ cells). The presence of CCL2 and CCL5 gave rise to macrophages (CCL2/5-MdM) with significantly up-regulated S100A8/A9 expression and significantly down-regulated CD613 expression. In CCL2/5-MdM the basal mRNA expression of CXCL1 and CXCL8 was significantly up-regulated. The mRNA expression of anti-inflammatory mediators (IL-10, Arginase) and pro-inflammatory mediators (IL-12 and iNOS) were not influenced by CCL2 and CCL5 during the differentiation and could not be enhanced by LPS stimulation.

Conclusions: The principal possibility of directing monocyte/macrophage differentiation *in vitro* has been demonstrated with rboCCL2 and rboCCL5, which skew the differentiation of monocytes into $S100A8/A9^{+}$ macrophages. Their LPS-insensitive phenotype is in contrast to the general opinion, that such macrophages *in vivo* are pro-inflammatory. Their *in vivo* role and function should be reconsidered and analyzed in more detail.

P1414

Equine arteritis virus replication in monocytic cells suppresses differentiation and function of dendritic cells

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Purpose/Objective: Equine viral arteritis (EVA) is an infectious disease of equids caused by equine arteritis virus (EAV), a single-stranded RNA virus of the family *Arteriviridae*. Dendritic cells (DC) are important modulators of the immune reaction with the ability to present antigen to naïve T cells and are crucial for the establishment of protective antiviral immunity. DC can be generated *in vitro* from monocytes (MoDC). Little is known of the effect EAV has on host immune cells, particularly DC. Understanding how the role of DC is affected during infection with EAV can provide insight into the mechanisms used by this virus to potentially exploit DC.

Materials and methods: An optimized system was established to generate equine monocyte-derived DC (eqMoDC) *in vitro*. Purified recombinant equine cytokines (GM-CSF & IL-4) were used to differentiate monocytes to immature MoDC (iMoDC) and a pro-inflammatory cocktail was used for maturation of iMoDC to mature MoDC (mMoDC). The phenotype of DC was studied, using flow cytometry and functional assays such as endocytosis/phagocytosis and mixed leukocyte reaction (MLR) were applied to characterise immature and mature MoDC. To study the interaction with EAV, MoDC were infected at multiplicity of infection of 5 with EAV strains of different genotypes and pathogenicity. Virus replication was determined through titration and real-time PCR (qPCR).

Results: Real-time qPCR and viral titrations revealed that EAV replicates in monocytes and MoDC. The replication was most efficient in mMoDC, but variable between the strains. Only the virulent strain caused a significant down-regulation of CD14 and CD163 on monocytes and of CD83 on mMoDC. The replication of the virus resulted in an apoptosis mediated cell death, which inevitably inhibited the differentiation and long-term function of DC. Functional studies carried out early after infection showed that EAV inhibited the endocytic and phagocytic capacity of Mo and mMoDC with minimal effect on iMoDC. In line with the detrimental effects and results of the virus replication, particularly infected mMoDC showed a reduced ability to stimulate T cells.

Conclusions: EAV evades the host antiviral immunity both by inhibition of antigen presentation early after infection and through killing infected DC during replication.

P1415

Expansion of combinatorial primary antibody repertoire in domestic cattle prior to the exposure of external antigens

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Purpose/Objective: In many domestic species fetal and neonatal gut-associated lymphoid tissue (GALT) is essential for the generation of the primary antibody repertoire. We found limited range of immunoglobulin genes in the bovine genome (Ekman *et al*. 2009, Niku *et al*. 2012) that effectively limits the degree of combinatorial diversity in cattle. In some species mechanisms such as junctional diversity may contribute significantly to the repertoire expansion. Terminal deoxynucleotidyl transferase (TdT) adds N-nucleotides between immunoglobulin gene segments primarily during heavy-chain gene rearrangement. However, in order to effectively compensate the limited recombinatorial diversity, many domestic species rely on GALT for further postrecombinatorial inflation of their primary antibody repertoire.

Somatic hypermutation (SHM) and gene conversion (GC) have been suggested to expand the limited antibody repertoire in ruminants prior to encountering external antigen. These post-recombinatorial processes are dependent on activation-induced cytidine deaminase (AID). It favours to deaminate C residues in WRCY hot spot motif (W = A/T, R = A/G, Y = C/T). This error in DNA sequence is either replicated or recognized by base excision or mismatch repair pathways. The outcome is often a mutation regardless of the mechanism.

Materials and methods: We studied the contribution of TdT and AID to the size of primary antibody repertoire in bovine fetuses. The expression levels were measured using reverse transcription-qPCR and the specific localizations were analyzed using immunohistochemistry. Several IgH cDNA libraries of different developmental stages were constructed and mutations were analyzed.

Results: Our work demonstrates TdT expression in bone marrow and a strong AID expression in ileal Peyer's patch. V-D-J junctions appeared heterogeneous in sequence, suggesting the addition of N-nucleotides by TdT. AID hotspot motif WRCY was preferentially mutated. Also, we found preferentialtargeting of complementarity-determining region (CDR) over framework region (FR) and preference of replacement over silent mutations in CDRs but not in FRs.

Conclusions: In conclusion, our results suggest that both TdT-mediated junctional diversity and AID mediated SHM contribute to the expansion of primary antibody repertoire in bovine fetuses prior to the presence exogenous antigens.

P1416

Extension of the storage time of blood in interferon gamma assays to diagnose paratuberculosis: combination of IL-7 and IL-12 stimulation

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Purpose/Objective: Detection of specific interferon gamma (IFN-γ) responses can aid in the diagnosis and control of paratuberculosis in sheep and cattle. IFN-γ is produced as part of the cell-mediated immune response to *Mycobacterium avium* subspecies paratuberculosis

(MAP) infection. IFN-γ detection assays offer the potential to detect more infected animals at an earlier stage of the disease than antibody ELISA, providing an opportunity for control strategies aimed at the removal of young infected animals before they shed bacteria into the environment. A limitation to the widespread application of IFN-γ assays has been logistical difficulties, as the assay needs to be performed within 8 h of blood collection. Earlier research has shown that, for bovine blood samples with a delayed assay setup (24 h), addition of Interleukin (IL)-12 can rescue the Th1 cells that produce IFN-g. For some countries such as Australia, however, samples may take up to 2 days to reach a laboratory. For this reason an improved protocol, applicable across multiple species, was required to increase white blood cell survival. IL-7 is a survival factor required to maintain resting T cells in cell culture. We hypothesised that IL-7 alone or in combination with IL-12, added at stimulation, could extend blood storage time.

Materials and methods: Blood collected from MAP infected sheep was stored from 0 to 4 days at room temperature, after which time IL-12 and or IL-7 was added to the blood with MAP specific antigens. After 48 h incubation the supernatant was harvested and examined by ELISA for IFN-γ.

Results: The addition of IL-7 and IL-12 in combination had a synergistic effect, giving IFN-g responses greater than adding IL-12 alone, for sheep blood stored up to 2 days. Better recovery of IFN-g responses was achieved for animals with low grade and paucibacillary lesions compared to animals with multibacillary disease. From a cohort of naturally infected sheep it was found that the same number of animals could be identified as test positive with blood samples stored for 2 days with addition of IL-7 and IL-12 compared to the same blood samples set up within 8 h of collection without additives.

Conclusions: This practical and easily implemented potentiation protocol (IFN-γPlus assay) extends the permissible transit time of blood samples from farm to laboratory for IFN-γ testing to detect Johne's disease.

P1417

FAS and FAS-L expression and apoptosis in the CD4+ T and CD8+ T cells from PBMC and spleen in dogs with visceral leishmaniasis

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Purpose/Objective: Dogs are the main domestic reservoirs of *L. (L.) chagasi*. Once in the vertebrate host, the parasite may cause visceral leishmaniasis, which can also be transmitted to humans. Infected symptomatic dogs show a reduction in T lymphocytes in spleen and peripheral blood. To investigate whether apoptosis is involved with diminished T cell counts in peripheral blood and spleen, apoptotic CD4+ T and CD8+ T cells from the spleen and peripheral blood of dogs naturally infected with *L. (L.) chagasi* and presenting clinical manifestations were quantified and compared with healthy dogs. The receptors involved in apoptosis were also investigated to determine which receptors are involved in the process of apoptosis.

Materials and methods: Fifteen symptomatic adult dogs infected by *L. (L.) chagasi* and six healthy dogs from a nonendemic area (controls) were included in the study. Samples from spleen and peripheral blood were used to quantify apoptosis in CD4+ and CD8+ lymphocytes and the cellular receptors involved in apoptosis. The apoptosis was measured by flow cytometry using Anexin V kit. The cellular receptors in CD4+ or CD8+ lymphocytes were measured using monoclonal antibodies anti-FAS-PE-Cy5, or anti FASL-PE, or anti TRAIL-PE by flow cytometry; the results were compared using the Mann Whitney test.

Results: The percentage of total of apoptosis levels in T CD4+ and T CD8+ lymphocytes from spleen and PBMC were higher in infected dogs than in controls. It was observed a decreased expression of FAS and FAS-L on CD4+ T lymphocytes from spleen from infected dogs compared to healthy ones, and similar result was observed to FAS-L on CD8 T lymphocytes from spleen. In PBMC only FAS-L on CD4+ lymphocytes showed decrease in infected dogs. The TRAIL receptor not showed difference between two groups. The blocking of receptors using monoclonal antibodies is in progress to understanding of the mechanisms of apoptosis.

Conclusions: Taken together, these data indicate that in infected dogs, the immunosuppression associated with chronic infection and decrease of lymphocytes is related to apoptosis of CD4+ and CD8+ T The present results could contribute to improving current understanding of the immune response in dogs infected with *L. (L.) chagasi*, while additional studies can allow future therapeutic interventions in order to reduce the depletion of lymphocytes, thus increasing the capacity of the defense.

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P1419

Genetic basis of resistance to viral diseases in rainbow trout

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Purpose/Objective: Controlling health of livestock is a key component of competitiveness and sustainability of fish breeding. Selection schemes (including marker-assisted selection) for resistance to pathogens already exist in fish farms. The aim of this project is to better understand the mechanisms of resistance to viral hemorrhagic septicemia virus (VHSV) in rainbow trout.

Materials and methods: Susceptible and resistant homozygous mitogynogenetic grand-parents were selected for their ability to support *in vitro* viral growth on fin explants, that was previously shown to correlate with resistance in order to study the genetic bases of resistance to VHSH. In parralel, cell lines were derived from a set of previously established resistant and susceptible mitogynogenetic clonal trout lines (Quillet *et al.*, 2007).

Results: A major quantitative trait loci (QTL) was identified, that controls both survival after waterborne infection and *in vitro* viral growth in fin explants. In parralel, the susceptibility of the cell lines to VHSV appeared well-correlated to the survival of the corresponding fish. The comparison of resistant versus susceptible lines demonstrated that key mechanisms are induced by the very early steps of infection, before the interferon induced response.

Conclusions: Taken together, these results strongly suggest the involvement of non-adaptive immune mechanisms in resistance to VHSV in rainbow trout.

Mechanisms and genes responsible for these phenotypes are under characterization, and will provide useful tools to develop selection schemes for future breedings.

Reference:

1. Quillet E *et al.*, FSI. 2007 May; 22(5):510–9. Epub 2006 Jul 31.

P1420

Identification of a parasite antigen recognised by both CD4 and CD8 T cells specific for lymphoblasts infected with *Theileria parva*

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Purpose/Objective: Infection of cattle with the tick-borne protozoan *Theileria parva* induces strong CD4 and CD8 T cell responses specific for parasitized cells. Adoptive cell transfer experiments with purified T cell subsets have demonstrated that CD8 T cells play a key role in

immunity and a number of *T. parva* antigens recognised by CD8 T cells have been identified. The aim of this study was to identify antigens recognised by parasite-specific CD4 T cells in order to investigate and the pathways by which the antigens are processed within infected cells and their role in immunity.
Materials and methods: Parasite-specific CD4 and CD8 T cell lines specific for parasitized cells were generated from cattle of defined MHC genotypes that had been immunised with *T. parva* by infection and treatment. CD8 T cells were used to screen a *T. parva* cDNA library as described previously (Graham *et al.*, 2006, *Proc Natl Acad Sci USA* 103: 3286-91). CD4 T cell antigen recognition involved screening of antigen-presenting cells incubated with pooled overlapping peptides for a series of candidate genes. T cell clones were generated to identify epitopes and to determine the dominance within the cultures.
Results: A novel CD8 T cell antigen termed Tp9 was identified, which was presented by the N*2301 class I MHC allele. A single 10 amino acid epitope was identified within Tp9. Screening of pooled peptides for 20 *T. parva* antigens with CD4 T cells identified four antigens including Tp9, which was recognised by CD4 T cells from seven cattle. A number of animals expressing different DR alleles did not recognise this antigen. Subsequent class II MHC typing showed that six of the seven positive animals shared the 1101 class II DRB allele and that they all recognised a single common peptide in Tp9. The CD4 T cell lines were also shown to recognise CHO cells transfected with a DR $\alpha\beta$ cDNA construct expressing the 1101 DRB allele. Analysis of a set of CD4 T cell clones from an animal homozygous for DRB 1101 revealed that >40% of the clones were specific for the epitope within this peptide.
Conclusions: The results of this study indicate that, as shown previously for CD8 T cells, CD4 T cell responses to *T. parva*-infected cells in individual animals are focused on a limited number of antigens/epitopes. Further studies are underway to investigate processing of the Tp9 antigen in infected cells for CD4 T cell response.

P1421

Identification of new antigens from *Rhipicephalus microplus* ticks associated to infestation phenotypes of susceptibility or resistance in bovine hosts

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Purpose/Objective: *Rhipicephalus microplus* cattle tick causes great economic losses to livestock. Current vaccines have partial and transient effects against infestations. To develop effective anti-tick vaccines, new targets must be identified. Bovines express breed-specific, heritable, contrasting phenotypes during infestation. Composition of tick saliva proteins may be affected by these different levels of host immunity and may be crucial to hematophagy, i.e., potential antigens.
Materials and methods: With DIGE, MudPIT and 454-based RNA-Seq we investigated the protein expression profile of salivary glands from nymphs (NSG), males (MSG) and females (FSG) from *R. microplus* ticks fed on resistant or susceptible bovines as well as unfed larvae (UFL) from eggs of females fed on these hosts.
Results: We identified 321 different proteins: 68 in samples derived from the ticks fed on susceptible hosts, 17 only on resistant and 236 shared by both groups. DIGE results showed 20 differentially expressed proteins in UFL, 27 in NSG and 35 in FSG. In addition, the sialotranscriptome revealed 11 676 coding sequences (CDS), with 3590 CDS for putative secreted proteins. Many differentially expressed proteins in the global analyses show similarity with proteases, nucleases, protease inhibitors, antimicrobial peptides and pathogen recognition proteins among others and are associated with the immunity raised in susceptible or resistant hosts.
Conclusions: This study represents the first attempt to identify protein profiles in developmental stages of *R. microplus* ticks that are affected by the different host immune responses developed by susceptible or resistant bovines. It offers an opportunity to identify new protective antigens for an effective anti-tick vaccine.

P1422

Ig-free colostral whey-supplemented formula: effects on adherent intestinal microflora and immune state of normal and low birth weight piglets

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Purpose/Objective: The use of hyperprolific hybrid sows in pig industry has led to increased litter sizes and consequently more low birth weight (LBW) piglets. At birth these animals are less competitive, which results in lower colostrum intake, making them more prone to infectious diseases. Pig farmers often transfer piglets to brooders where they are artificially fed with formula *ad libitum* until weaning. This research aims to elucidate the benefit of colostral whey fraction provided in conjunction with formula by its effect on the piglet's gut immune system and intestinal flora.
Materials and methods: LBW and normal birth weight (NBW) piglets, from day 3 onwards, were fed formula or formula supplemented with colostral whey fraction (Ig-free, <50 kDa, daily/7d). At 10 or 28 days of age, ileal samples were collected for analysis of the adherent microflora (LPS and Gram staining) combined with RT-PCR analysis of (innate) immune-related genes (TLR pathway, cytokines).
Results: In contrast to sow-fed animals, which had a beneficial ratio (i.e. 1.5) of $Gram^+$ to $Gram^-$ bacteria, LBW and NBW piglets reared on formula till day 10 had an adherent flora that was richer in $Gram^-$ bacteria (ratio's 0.66 and 0.33, respectively). Alternatively, formula feeding supplemented with colostral whey fraction, improved these ratio's (0.75 and 1.33, respectively). Whey fraction-supplemented piglets showed a significantly increased TLR-2 expression ($P = 0.01$) and lower expression of IL-1b ($P = 0.04$) in the gut, irrespective of birth weight. Additionally, NOD2 expression was increased specifically in LBW piglets when fed supplemented formula ($P = 0.05$). At day 28, most animals displayed a predominantly $Gram^+$ adherent microflora (ratio >1), except for NBW piglets fed supplemented formula (0.6). However, the optimal ratio of a sow-fed animal (LBW: 1.875, NBW: 2.5) was never reached. The expression of virtually all immune proteins dropped with age, except for NF-κB in animals fed supplemented formula ($P = 0.03$), which is consistent with the maturation of the immune system towards adaptive immunity.
Conclusions: In conclusion, a more beneficial microflora is preponderant in piglets fed formula with colostral whey fraction at day 10, which seems to be related to an altered immune state of the gut. However, this effect did not prevail in the long term, possibly due to supplementation restricted to the first week.

P1423

Immunoproteomics analysis of Brucella abortus antigen reveals differential antibody profiles between S19-vaccinated and naturally infected cattle

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Purpose/Objective: Brucellosis is an infectious disease caused by bacteria of the genus *Brucella* and it is one of the most widespread zoonotic diseases, including infectious abortion in domestic animals and potentially debilitating infection in humans. The food-producing herds, such as cattle, sheep, goats, and pigs are highly susceptible to disease. In some areas, however, the complete control of disease is complicated by the presence of wildlife reservoirs. The diagnosis of brucellosis is mainly based on serological methods, because of the requirement for faster and more reliable diagnostic tests. The majority of the conventional serological tests use whole cell preparations, sonicated cell extracts, or lipopolysaccharide (LPS)-enriched fractions obtained from smooth (S) *B. abortus* strains, called the S-LPS antigen. Although LPS induces a strong antibody response and classical serological tests are mainly based on the detection of antibodies to LPS, there are some limitations in the use of these S-LPS antigen preparations.

This study aimed to characterize a *B. abortus* S19 antigen preparation obtained by Triton X-114 (TX-114) extraction through immunoproteomics to differentiate infected from vaccinated cattle.

Materials and methods: Three groups of bovine were studied: GI, 30 naturally infected cows; GII, 30 S19-vaccinated heifers; and GIII, 30 nonvaccinated seronegative cows. Serum samples from these animals were analyzed and one-dimensional (1D) and two-dimensional (2D) electrophoretic were obtained from the TX-114 hydrophilic phase antigen of the bacteria, followed by MS/MS analysis, after immunoblotting assays.

Results: The results revealed a broad spectrum of polypeptides, varying from 10 to 79 kDa. 1D immunoblot showed widespread seroreactivity profile in GI compared with restricted profile in GII. Three antigenic components (10, 12, 17 kDa) were recognized exclusively by GI sera, representing potential markers of infection and excluding vaccinal response. The proteomic characterization revealed 56 protein spots, 27 of which were antigenic spots showing differential seroreactivity profile between GI and GII, especially polypeptides <20 kDa that were recognized exclusively by GI. MS/MS analysis identified five *B. abortus* S19 proteins (Invasion protein B, Sod, Dps, Ndk, and Bfr), which were related with antigenicity in naturally infected cattle.

Conclusions: In conclusion, immunoproteomics of this new antigen preparation enabled the characterization of proteins that could be used as tools to develop sensitive and specific immunoassays for serodiagnosis of bovine brucellosis, with emphasis on differentiation between S19 vaccinated and infected cattle.

P1424

Immunoreactivity to salmonella infection in chicks protected with enteroccocus administration

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Purpose/Objective: The aim of study was to determinate in *Salmonella* Enteridis PT4 infected chicks, protected with 7-day *Enterococcus faecium* EK 13 administration, the influence on some immunocompetent cells in blood and immune organs (thymus, bursa of Fabricius, spleen).

Materials and methods: Sixty 2-day-old White Plymouth Rock chicks were divided into four groups * control (C), administered with *E. faecium* (EF; 1.10^{11} CFU), infected at day 7 with *S. Enteritidis* PT4 (SE; 1.10^{8} CFU), and combined EF + SE. The samples from five chicks of each group were taken on days 2, 5 and 7 after salmonella infection. Isolated lymphocytes, flow cytometry, indirect immunoflourescent method and primary mouse anti-chicken monoclonal antibodies (CD3, CD4, CD8, IgM) were used to measure relative percentage of positive-stained cells. The values of white blood cells were tested haematologically.

Results: The increase of lymphocytes, CD3+ and IgM+ cells on 7 days post salmonella infection (pi) in blood was observed in combined EF + SE group compared to SE and C groups. Relative percentage of CD3+ and CD4+ cells in EF + SE group was improved in spleen on days 2 pi. Thymus showed increase of CD3+, CD4+ and CD8+ cells on days 5 pi. No significant changes were observed in bursa of Fabricius.

Conclusions: The results showed higher values of T lymphocyte subpopulations in EF + SE group during the experiment. Their increase depicted the important of cell-mediated immune response to salmonella infection. Administration of *E. faecium* EK13 showed beneficial effect which led to earlier and more intensive T cell immune response in comparison to salmonella infection alone and may be used as probiotic additive in food.

P1425

Infection by swine, human or avian influenza virus differentially activates porcine dendritic cells cytokine profile

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Purpose/Objective: To study the porcine immune response generated by porcine bone marrow derived dendritic cells (poBMDCs) against swine, human and avian influenza viruses *in vitro*.

Materials and methods: *In vitro*, poBMDCs were infected with 10^4 $TCID_{50}$ of circulating trains of swine influenza virus (SwIV) A/Swine/Spain/SF32071//2007 (H3N2), 2009 human pandemic influenza virus A/Catalonia/63/2009 (H1N1), low pathogenic avian influenza virus (LPAIV) A/Anas plathyrhynchos/Spain/1877/2009 (H7N2) or high pathogenic avian influenza virus (HPAIV) A/Chicken/Italy/13474/99 (H7N1) for 4, 8, 16 and 24h. Supernatants were collected for IFN-α,

TNF-α, IL-12 and IL-18 detection by ELISA, while RNA was extracted from the cells to analyse the gene expression of *NF-κB*, *TGF-b*, *IL-10* and *IFN-b* by RT-qPCR. Moreover, after inhibition of NF-κB with CAPE, cells were infected with the swH3N2 influenza virus and IL-12 and IFN-α secretion was evaluated by ELISA.

Results: All viruses induced expression of *NF-κB*, *TGF-β* and *IL-10* in swine poBMDCs to different extents and in a time dependent manner suggesting a specific function in cytokine production. All viruses induced the secretion of IL-12 mostly at 24 hpi and IL-18 which was time dependent. Only swH3N2 induced IFN-α in a time dependent manner. Swine H3N2, aH7N2 and aH7N1 induced secretion of TNF-α also in a time dependent manner. Inhibition of *NF-κB* resulted in a decrease of IFN-α and IL-12 by swH3N2 infected-poBMDCs at 24 hpi, suggesting a role of this transcription factor on the synthesis of these cytokines.

ConclusionsThere is a differential response of porcine DCs against swine, avian or human influenza virus. These data pave the way for understanding the relation between influenza viruses and porcine DCs in triggering the immune response in swine through soluble mediators such as cytokines.

P1426

Influences of birth weight, age and milk composition on the developing porcine immune system

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Purpose/Objective: Introducing hyperprolific hybrid sows in pork production has resulted in large litters that contain up to 20% low birth weight (LBW) piglets. As these piglets are more susceptible to disease than normal birth weight (NBW) piglets, the litter is often weaned early and subsequently fed with formulated milk (formula). Although this strategy allows LBW piglets to catch up growth, their morbidity and mortality do not decrease. This could be due to the fact that formula is low in bioactive molecules (e.g. growth factors, hormones and cytokines), necessary for the development of the immune system. This study determined the influences of birth weight, age and milk composition on the development of the porcine GALT during the normal suckling period.

Materials and methods: To this purpose, LBW and NBW piglets were pre-weaned at the age of 3 days, and subsequently fed *ad libitum* for 7 or 25 days with either formula or formula supplemented with a whey fraction (FW) (Ig-free, molecular weight <50 kDa) that was daily administered via gavage until day 10. Control animals suckled the sow. After euthanasia of the animals at day 10 or 28, samples of the ileum were processed for quantitative analysis of $CD4^+$, $CD8^+$ and $CD172a^+$ cells that were immunohistochemically visualized.

Results: With increasing age, the lower $CD4^+$ and $CD8^+$ densities in sow-fed LBW piglets, compared to NBW piglets, were leveled up by both types of artificial feeding. At day 10, the densities of lymphoid cells in the formula-fed piglets resembled most those present in the control piglets when compared to piglets fed FW. This means that formula feeding results in lower immunological stress, i.e. altered numbers of lymphoid cells, caused by pre-weaning at 3 days of age. As a result, the higher immunological stress in the FW-fed piglets seems to be caused directly by the whey fraction and/or the manipulation of daily gavage. Elevated $CD4^+$ and $CD8^+$ cell densities in the lamina propria were seen in the formula-fed group at day 28 compared to day 10. In contrast, only the $CD4^+$ or $CD8^+$ cell populations increased in the FW and sow groups, respectively.

Conclusions: It is concluded that FW-fed piglets display an altered development of the GALT, as demonstrated by the inverted $CD4^+$/$CD8^+$ ratio compared to sow- and formula-fed animals. Consequently, formula feeding could be a valuable alternative for suckling the sow until conventional weaning age.

P1427

Investigation of the pathogenic effects and specificity of alloantibody in Bovine Neonatal Pancytopaenia (BNP)

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Purpose/Objective: Bovine Neonatal Pancytopaenia (BNP) is a recently emerged haemorrhagic disease of calves characterised by leukocyte depletion and thrombocytopoenia, which has been linked epidemiologically to the use of a vaccine with a novel adjuvant. Feeding colostrum from cows that have previously produced a BNP affected calf has been shown to induce the disease in some calves, suggesting BNP is mediated by alloantibodies. Herein we report the results of an experimental study to define the haematological changes in calves fed colostrum from dams of BNP calves and present preliminary findings on the specificity of the alloantibodies present in dam serum.

Materials and methods: Two groups of five calves that received a standardised colostrum pool from known BNP dams or an equivalent colostrum pool from non-BNP dams, were subjected to detailed clinical, haematological and pathological analysis, including examination of bone marrow biopsies. The alloreactivity of BNP dam serum was tested using a panel of cell lines of defined MHC genotypes. Specificity was investigated further using tranfected lines expressing individual class I and class II MHC alleles, and mutated cell lines lacking expression of class I MHC alleles.

Results: All challenge calves showed bone marrow and haematological changes consistent with the development of BNP, supporting the hypothesis that BNP is induced by ingestion of colostrum-derived alloantibody. Haematology results coupled with serial bone marrow histopathology results indicated that alloantibody damage to different blood lineages varies. Peripheral cell depletion was confined to leukocytes and platelets, while bone marrow damage affected the primitive precursors and lineage committed cells of the thrombocyte, lymphocyte and monocyte lineages but only to more primitive precursors in the neutrophil, erythrocyte and eosinophil lineages. Serum from BNP dams was shown to have a higher titre of lymphocyte reactive antibodies than control cows and showed variable patterns of alloreactivity against a panel of MHC-defined cells.

Conclusions: BNP is caused by the ingestion of colostral alloantibody which produces variable damage to different haematopoietic lineages. Ongoing studies aim to determine the antigenic specificity of the antibodies in relation to their differential effects.

P1428

Proteome of Brucella abortus 2308 and its virB type IV secretion system mutant

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Purpose/Objective: Brucellosis is a zoonotic disease caused by the genus *Brucella*, that is an alpha-2 proteobacteria with a type IV secretion system (T4SS) known as VirB. The T4SS is necessary to gain virulence by building up a replicative vacuole associated with the endoplasmic reticulum of the host cell. Since the mutant strains do not possess a T4SS, they are unable to secrete the proteins usually produced and secreted for the wild type strains. A different protein profile in the mutant strain suggests that the proteins identified may be T4SS-related and they can be used as a biomarker of the infection.
Materials and methods: A *virB* T4SS mutant of the *B. abortus* 2308 strain and its wild-type strain were grown in acid medium. The protein content of the bacteria was analyzed by 2D-PAGE and mass spectrometry. STRING program v8.3 was used for known interactions and possible predictions among proteins including direct (physical) and indirect (functional) interactions.
Results: A total of 47 overexpressed and 22 underexpressed proteins from the *virB* T4SS mutant strain were selected and sequenced. The majority of the 69 analyzed proteins have not been described before either as over or under-expressed in relation to a *virB* T4SS mutation, whereas some of them have been already described by other groups as potentially important secretory proteins in other *Brucella* species.
Conclusions: An important number of the proteins identified are outer membrane and periplasmic space protein, which makes them particularly important new T4SS-related candidate proteins. We confirm that the T4SS regulates a large number of proteins with diverse functional categories that can be used as biomarkers of the infection.

P1429

Synergic effects of canine IL-2 and IL-7 genes on enhancing immunogenicity of canine parvovirus VP2 gene vaccine in mice

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Purpose/Objective: To investigate the effects of canine interleukin-2 (cIL-2) and cIL-7 genes on enhancing the immunogenicity of canine parvovirus (CPV) VP2 DNA vaccine.
Materials and methods: The bicistronic vectors of cIL-2 and cIL-7 genes was constructed using the eukaryotic expression vector containing internal ribosome entry site (IRES). The cIL-2/clL-7 dicistronic vector plus previously constructed vectors, including CPV VP2 DNA vaccine vector, cIL-2 vector and cIL-7 vector, were used to co-immunize the mice with the different combinations, consisting of VP2 alone, VP2+cIL-2, VP2+cIL-7 and VP2+cIL-2/cIL-7. The VP2-specific antibody levels in immunized mice were measured by ELISA at different time post-immunization. The proliferation indexes and interferon-γ expression were measured by lymphocyte proliferation assay and ELISA, respectively.
Results: The results showed that the cIL-2/cIL-7 bicistronic vector was correct and could mediate cIL-2 and cIL-7 gene expression in eukaryotic cells. Immunization results revealed that the antibody titers and the neutralizing antibody levels of the mice co-immunized with VP2+cIL-7/cIL-2 vectors were significantly higher than that with either VP2+cIL-2 vectors or VP2+cIL-7 vectors ($P < 0.05$). The lymphocyte proliferation indexes of VP2+cIL-7/cIL-2 vector-immunized mice were also higher than that of other two groups although not statistically significant. However, the IFN-γexpression levels of VP2+cIL-7/cIL-2 vector-immunized mice were significantly higher than other immunized mice ($P < 0.05$).
Conclusions: cIL-2 and cIL-7 genes have the significant synergic effects on enhancing the immunogenecity of CPV VP2 DNA vaccine.

P1430

The biological characterisation of avian receptor activator of NF-κB ligand and RANK

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Purpose/Objective: The Th1/Th2 paradigm is well-established, both in mammals and in the chicken, as a model for the initiation of appropriate adaptive immune responses to clear infections with intracellular and extracellular pathogens respectively. Cytokines play a pivotal role in the coordination of these responses. In the chicken, detailed knowledge of the initiation, control and potentiation of the adaptive immune response is lacking compared to mammals, and the immune system is somewhat different. For example, chickens have a reduced number of genes in their MHC, lack lymph nodes and have a separate organ and mechanism for development of the B cell receptor (antibody) repertoire. The availability of the avian genome sequence has enhanced our ability to identify avian immune genes and subsequently to investigate their biological roles. Receptor activator of NF-κB ligand (RANKL) and its receptor, RANK, are members of the tumour necrosis factor superfamily which play pivotal roles in processes of immunity, differentiation and apoptosis. In mammals, RANKL and RANK are transmembrane proteins present on the surface of Th1 cells and dendritic cells (DC) respectively. Their interaction induces the binding of a number of adaptor proteins called tumour necrosis factor associated factors (TRAFs) to the intracellular domain of RANK, inducing the expression of pro-inflammatory cytokines and enhancing the life span of DC by the upregulation of anti-apoptotic molecules.
Materials and methods: Both genes have been identified in the avian genome and cloned along with TRAF 2, 3, 5 and 6. Chicken RANKL and RANK soluble fusion proteins have been generated from COS-7 cells and verified by western blot analysis and ELISA.
Results: ChRANK, which is predicted to be 655 amino acids in length, consists of four cysteine-rich domains, contains three putative TRAF-binding domains near the COOH-terminus that are conserved within mammalian RANK. However, chRANK seems to be missing a TRAF-binding motif expressed in the middle of the intracellular domain (342−349) which is one of the sites for TRAF2 binding but not essential for RANK signalling in human and mouse.
Conclusions: We were the first to successfully generate non-mammalian bone-marrow derived DC and thus now have the tools to study the biological role of avian RANKL/RANK in DC biology and Th1 responses in the chicken.

P1431

The cattle germline IGHV repertoire is very limited

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Purpose/Objective: Ruminants are thought to have a small germline repertoire of Ig gene segments, but the *IGHV* genes have been poorly known. We have now characterized the bovine *IGHV* repertoire based

on genome sequencing data. The expression and chromosomal localization of the identified genes were investigated and the repertoire was compared to related species.

Materials and methods: Both bovine genome assemblies (Btau_4.2 and UMD_3.1) and the raw sequencing data were blasted using *IGHV* sequences from ruminants, human and mouse. RSSs obtained from the identified genes were used to search for identical motifs. The functionality of the *IGHV* genes was analyzed *in silico* and utilizing sequences from fetal ileal RNA and the EST database. Phylogenetic analyses were supplemented with clan-specific genomic PCR. Chromosomal localization of the *IGHV* genes was investigated by FISH using BAC probes.

Results: A total of 36 *IGHV* genes were identified and assigned to three subgroups. In the fetal ileal cDNA library/EST database, 99%/94% of sequences aligned to these genes with 98.4/92.3% average identity. Only 10 genes appear functional, all in subgroup IGHV1, with identical FR and CDR lengths and canonical CDR structures. The functional genes are contained in homology units consisting of a subgroup IGHV1 gene and a subgroup IGHV2 pseudogene.

The bovine *IGHV* genes belong to mammalian clans I and II. No clan III genes were found in the sequence data or by clan-specific PCR. Of related species, sheep and elk were negative for clan III, but DNA from deer, pig and dolphin was amplified by clan III primers. The functional bovine subgroup is homologous to the murine IGHV2 (Q52), but there is no clear human homolog.

Most IGHV genes were found in UMD_3.1 but missing from Btau_4.2. In UMD_3.1, they were distributed in BTA21 and BTA7. FISH analysis confirms the locus on BTA21 but suggests that the BTA7 locus is an assembly error.

Conclusions: The bovine IGHV repertoire is very limited. The single functional subgroup was likely generated by recent duplication events. Within the related Cetartiodactyla, the evolution of *IGHV* gene usage appears highly dynamic. The complex IGH locus is largely incorrectly assembled in the current cattle genome versions. We are now sequencing *IGHV* genes in several individual animals to verify the repertoire and to evaluate the allelic diversity.

P1432

The effect of florfenicol and *E. coli* lipopolysaccharide interaction on lymphocytes subset in newly-hatched chicks

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Purpose/Objective: Florfenicol is a broad-spectrum bacteriostatic antibiotic that is widely used in poultry production. The aim of this study was to investigate the influence of florfenicol and *E. coli* lipopolysaccharide interaction on T and B cells subsets in lymphoid organs.

Materials and methods: One-day-old broiler breeder Flex were treated orally, into the crop with a single doses of 30 mg/kg during 5 days, whereas LPS (O127:B8, Sigma) was given i.v. once at the dose of 200 μg kg -1 BW on 2nd day of experiment (d.e.). The samples were done 24 h after last treatment and at 14th d.e. The effect of florfenicol on subset of T and B cells pretreated with high doses of *E. coli* LPS and non-treated LPS was evaluated by measurement percentage of chicken lymphocytes in thymus (CD3+, CD3+ TCR $\gamma\delta^+$, CD4+, CD4+CD8+, CD8+, TCR $\gamma\delta^+$ cells), spleen (CD3+, CD4+, CD8+, TCR $\gamma\delta^+$, Bu-1+ cells), and bursa of Fabricius (Bu-1+ cells) by flow cytometry using appropriate fluorescein and phycoerythrin conjugated monoclonal antibodies.

Results: Florfenicol decreased percentage of Bu-1+ cells in bursa of Fabricius on day 7. It was also demonstrated decrease percentage of CD3+ and CD8+CD4+ thymocytes. In spleen the decrease percentage of CD3+, CD4+ on 7th d.e. and CD3+, CD4+, CD8+, TCR $\gamma\delta^+$, Bu-1+ cells on 14th d.e were observed. Additionally, it was shown decrease percentage of TCR $\gamma\delta^+$ and Bu-1+ cells in spleen on day 7 and 14 in florfenicol with LPS treated group. However, increase percentage of CD8+ cells in thymus was observed in both florfenicol and florfenicol with high doses of LPS treated group on day 7.

Conclusions: This study provides new information which indicate the influence of florfenicol on percentage of T and B cells in lymphoid organs. This is especially important on account of the fact that normal function of this organs have an influence on development and differentiation of lymphocytes in birds. The suppressive effect of florfenicol on selected cells was shown. Moreover, this effect was enhanced by *E. coli* LPS. This could indicate that florfenicol tratment might impair the immune response to bacterial endotoxin in chicks.

P1433

The importance of myeloid differentiation factor 88 on the infestation by *Rhipicephalus sanguineus* ticks

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Purpose/Objective: Ticks are hematophagous arthropods that parasitize vertebrate host and transmit a variety of infectious agents to domestic animals, wildlife and humans. In the tick-host interface there is an involvement of both the innate and acquired immune response of the host, which are modulated by the inoculation of tick saliva. Our group has shown that mice are susceptible to tick infestations and develop a Th2 type immune response. Additionally, we and others have shown that tick saliva can modulate the function of various immune cells, including macrophages, lymphocytes, and modulate differentiation, maturation, migration and function of dendritic cells (DCs) stimulated with ligands for Toll-like receptors (TLRs). We observed that saliva of *R. sanguineus* induced a high expression of TLR-2 on the DCs surface *in vitro*. These results suggest that tick saliva possibly can modulate the host immune response through TLRs. Knowing that the Receptor adaptor myeloid differentiation factor 88 (MyD88) signaling is critical for most TLRs signaling, we investigated if this molecule interfere in the infestation with *R. sanguineus* on mice.

Materials and methods: C57/B6 WT and MyD88-/- mice were infested with three pairs of ticks and the biological parameters of the ectoparasites were evaluated, such as number and weight of engorged females; feeding period; egg mass weight; hatching rate and reproductive index.

Results: The number of engorged female ticks on MyD88-/- mice was significantly ($P < 0.05$) enhanced when compared with C57Bl/6 WT mice. Related to reproductive parameters, we observed a large difference within animals from the C57Bl/6 WT mice group, impairing the statistical comparison. New experiments are being done in order to enhance the animal's number.

Conclusions: Our findings demonstrate the importance of the MyD88 for the success of the *R. sanguineus* infestation, suggesting that tick infestation may modulate mice immune response through the TLR-MyD88 pathway.

P1434

The T cell immunoglobulin and mucin domain (Tim) family in a non-mammalian species – a unique isoform of Tim4

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Purpose/Objective: T cell immunoglobulin and mucin domain (Tim) proteins are a family of molecules that are expressed on specific cell types (e.g. Tim4 on dendritic cells and Tim1 on CD4+ Th2 cells). Their functions include enhancement of the phagocytosis of dead cells and co-stimulation of Th2 cells through Tim1-Tim4 interactions. In the chicken, there are only two Tim family molecules, Tim1 and Tim4 (compared to three in man and eight in mouse). Uniquely, chicken Tim4 has two isoforms (long and short) formed by alternative splicing of three exons leading to an extra immunoglobulin domain in the long isoform. The chicken Tim genes have been cloned, expressed and monoclonal antibodies have been raised to Tim1 and both isoforms of Tim4.

Phosphatidylserine (PS) is an indicator of apoptotic cells. In dying cells PS translocates from the inner layer of the cell membrane to the outer layer. As receptors for PS, TIM molecules enhance the clearance of dead cells by macrophages or dendritic cells. Here I will focus on their phagocytic functions in the chicken immune system.

Materials and methods: Dot-blotting.

Solid phase ELISA.

Flow-cytometary.

Phagecytotic activity assay.

Results: Recombinant chicken Tim molecules can bind PS, but not other phospholipids. Stimulated splenocytes express more Tim4S (short isoform) ligand. If PS is blocked, Tim4S proteins exhibit decreased binding to stimulated splenocytes. NHI-3T3 cells transfected with chicken Tim molecules have increased ability to phagocytose dead cells.

Conclusions: In conclusion, this study demonstrates that chicken Tim molecules play an important role in phagocytosis through interaction with their ligand * phosphatidylserine.

P1436

TLR2 and TLR4 expression and oxidative response in PBMC from dogs with visceral leishmaniasis

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Purpose/Objective: Leishmaniasis visceral is an endemic disease that has spread over several continents, mainly in tropical and subtropical regions. It is caused by *Leishmania chagasi* and affects millions people worldwide. The parasite is transmitted by sandflies, which infect mammals including man and dog, which is considered the most important urban reservoir of *L. chagasi*. Dogs with visceral leishmaniasis are highly susceptible to infection and Th2 immune response in target organs facilitates the spread of the parasite in the host. Toll-like receptors 2 and 4 were involved in *Leishmania* recognition, but the role of TLRs in dog pathogenesis of canine visceral leishmaniasis and the effect of the interaction with parasite in the antimicrobial activity has not been addressed.

Materials and methods: Thirty dogs presenting clinical symptoms compatible with leishmaniasis and positive detection of antibodies anti-leishmania were used, five healthy dogs were used as control. To examine the expression of TLR 2 and TLR 4 in PBMC of infected dogs with *L. chagasi* and controls, PBMCs were double-stained with specific fluorochrome-conjugated antibodies: FITC conjugated monoclonal antibody anti-human TLR2, and PE conjugated monoclonal antibody anti-human TLR4 or control isotypes. Intracellular ROS levels were measured in PBMCs using H2DCFDA (29,79-dichlorodihydroflurescein diacetate), according to manufacturer's intructions. After the acquisition of data in EasyCyte mini® (Guava, Hayward, CA, USA), the analysis of the data was performed in the Software Guava Express® Plus. The results were compared using nonparametric tests.

Results: Comparison between the groups showed a decrease of TLR4 on PBMC from infected dogs ($P < 0.05$), the TLR2 was equally express on PBMC from infected and control dogs. The ROS production was higher in PBMC from infected dogs compared to control dogs ($P < 0.05$).

Conclusions: The data reveals that the receptor TLR4 is involved in immune response in symptomatics dogs chronic infected. The high ROS production observed in infected dogs indicates that ROS not showed antimicrobial effect and suggest that ROS could be useful by the protozoa to chronic the infection. Studies are in progress to further determine the mechanisms involved in this process.

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Poster Session: Virus Infections

P1438
'Enforced virus replication' as an immunological mechanism for adaptive immune activation

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Purpose/Objective: The innate immune system suppresses virus replication by interferon type I (IFN-I) and also induces presentation of viral antigens to adaptive immune cells. We analyzed how the innate immune system manages to inhibit virus propagation but still allows the presentation of sufficient amounts of antigen to adaptive immune cells.
Materials and methods: Mouse model of VSV infection.
Results: We found that the expression of *Usp18* in metallophilic $CD169^+$ macrophages reduces their IFN-I responsiveness, thereby allowing locally restricted replication of virus. This early virus replication was essential for the induction of sufficient antiviral immune responses and, therefore, for inhibiting the spread of virus to neuronal tissue and preventing fatal disease.
Conclusions: In conclusion, we found that 'enforced' virus replication within marginal zone macrophages is a novel immunological mechanism that ensures the production of sufficient antigen for effective adaptive immune activation.

P1439
A crucial role for the CC-chemokine receptor CCR5 in the pathogenesis of dengue virus infection in mice

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Purpose/Objective: Dengue virus (DENV) infection is a public health problem faced by 2.5 billion people in tropical countries. Infection is characterized by a systemic inflammatory response and hematological alterations that may lead to shock and death in severe cases. Recent clinical data have shown an association between components of the chemokine network and severity of Dengue. Previous works from our group indicate that CC chemokine receptors play discrete roles in DENV infection in mice. The chemokine receptor CCR5 has been associated with severe disease following flavivirus infection. Here we investigated the role of CCR5 receptor in a mice model of DENV infection and disease.
Materials and methods: Wild-type (WT), $CCR5^{-/-}$ and WT mice treated with CCR5 antagonists were inoculated intraperitoneally with a mouse-adapted strain of DENV-2 (P23085). Samples were collected at day 5 and 7 post-infection (p.i.). Platelet count, hematocrit, cytokines (IFN-γ, TNF-α, IL-6, CXCL-1) and myeloperoxidase (MPO) activity were evaluated in spleen and liver, in addition to histopathology in the liver. Viral load was analyzed in organs, blood and in murine primary macrophages culture samples, treated or not with CCR5 antagonist Met-RANTES or Pertussis toxin (PTX).
Results: CCR5 deficiency or blockade with antagonists prevented dengue associated disease. All hematological parameters, MPO activity, tissue damage, cytokines and chemokines levels in organs largely affected by the infection were strongly reduced in $CCR5^{-/-}$ mice compared to WT at day 7 p.i. ($P < 0.01$). Viral load was reduced in blood and spleen of $CCR5^{-/-}$ mice at day 5 p.i. and completely abolished at day 7 p.i., contrary to the viral load observed in WT mice, which increased from day 5 to 7 p.i. ($P < 0.001$). $CCR5^{-/-}$ primary macrophages infected with DENV presented reduced viral load in cells compared to WT, which can be reproduced by treating WT cells with Met-RANTES or PTX.
Conclusions: The lack of CCR5 cannot prevent DENV infection *in vitro* and *in vivo* but can prevent disease. We hypothesized that the reduced viral load in mice organs leads to a reduced inflammatory response and tissue damage, allowing an effective viral clearance. We aim to investigate whether the diminished viral load in $CCR5^{-/-}$ tissues is due to impaired DENV entry into cells or impaired replication.

P1440
A novel subset of anti-viral T cells: the CD161dim $CD8^+$ population

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Purpose/Objective: $CD8^+$ T cells are important in the control of viral infections, and can be divided into three subsets based upon expression of the C-type lectin CD161. While the $CD161^{bright}CD8^+$ T cell population is largely comprised of anti-bacterial mucosal associated invariant T (MAIT) cells, the phenotype and function of the $CD161^{dim}$ $CD8^+$ T cell population, which can constitute up to 60% of the $CD8^+$ T cell pool, remains to be characterized. This $CD161^{dim}$ population is found in the naïve pool in cord blood, but in adult blood contains viral-specific cells, including those specific for epitopes from CMV, EBV and influenza, and is especially enriched in responses to Hepatitis B and C.
Materials and methods: We have used gene expression analysis and 'Cytometry by Time-of-Flight (CyTOF)', to probe the phenotype and function of these cells.
Results: They have a highly differentiated memory phenotype and share some features with the $CD161^{bright}$ population, including expression of the transcription factor ZBTB16 and the multi-drug efflux pump, MDR-1, albeit at lower levels. Functional expression of MDR-1 suggests that $CD161^{dim}$ $CD8^+$ T cells may be relatively resistant to cytotoxic agents and steroids. Expression of CD16 in this subset indicates additional functional capacity.
Conclusions: Overall, $CD161^{dim}$ $CD8^+$ T cells constitute a potentially important population in the context of viral infection, especially hepatic infection, with distinct phenotype and function.

P1441
Activation of TLR-2 by murine hepatitis virus (MHV) induces exacerbated inflammatory response in liver increasing hepatic damage

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Purpose/Objective: Viruses are generally detected by intracellular Toll-like receptors (TLRs) and helicases that sense nucleic acids and trigger signalling pathways leading to antiviral interferon (IFN) α, β production, but evidences show that surface TLRs may also participate in mounting an initial acute inflammatory response. It was recently reported that viral proteins from hepatitis viruses (HVC and HVB) could activate TLR-2 and -4-mediated signalling, however the consequences on hepatitis outcome remain unknown. We have reported that the S protein of the highly virulent mouse hepatitis virus 3 (MHV3) was able to ligate TLR-2 on macrophages, increasing inflammatory cytokine response. Using different virulent MHV viruses, we investigated the *in vivo* role of TLR-2 in viral replication, IFNs and inflammatory responses in the development of a fulminant hepatitis.

Materials and methods: Wild type and TLR2$^{-/-}$ C57BL/6 mice were infected with a high-, mild- or low-pathogenic MHV (L2-MHV3, MHV-A59 and YAC-MHV3 respectively). At various times postinfection, livers were fixed in formaldehyde or RNA and protein were extracted for qRT-PCR and/or ELISA assays to evaluate the expression of viral nucleocapsid, CEACAM1a, TLRs, IFNα, β, γ, IRFs, and inflammatory cytokines and chemokines. Blood was collected for serum determination of ASAT and ALAT levels.
Results: Results showed that extensive hepatic damage occurred sooner in L2-MHV3-infected mice and correlated with viral nucleocapsid mRNA levels. Surprisingly, TLR-2 expression levels strongly only increased in the liver of L2-MHV3-infected mice while no marked differences were noted for other TLRs nor for the viral receptor CEACAM1a. IFN-b production in the liver of L2-MHV3-infected mice was delayed when compared to MHV-A59, whereas inflammatory cytokine and chemokine levels were higher in L2-MHV3-infected mice. Most of these changes were associated to TLR-2 since hepatic lesions, viral replication, IFN-b and inflammatory cytokine and chemokine levels strongly decreased or were delayed in TLR-2$^{-/-}$ mice infected with L2-MHV3.
Conclusions: These results reveal for the first time that virulence of hepatotropic viruses may be related to their ability to activate the TLR-2 pathway resulting in an exacerbated inflammatory response increasing hepatic damages. Blocking of TLR-2 pathway may be considered as a new potential therapeutic approach in controlling viral-induced hepatitis.

P1442
Age-dependent seroprevalence to influenza A(H3N2) variant virus in 2011 – comparison to immunity to seasonal influenza A(H3N2) circulating in the mid-1990's

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Purpose/Objective: Since August 2011, 13 cases have been identified in the US of human infection with a variant of influenza A(H3N2) virus, A(H3N2)v, that is circulating in North American pigs and that descends from human seasonal H3N2 viruses circulating in the mid-1990s. Almost all cases occurred in children. In some no exposure to pigs were recognised and limited human-to-human transmission appears to have occurred. To assess the risk of further spread of the influenza A(H3N2)v virus in humans, the immunity to A(H3N2)v has been investigated and compared to seroepidemiological data from 1996 to 1998.
Materials and methods: Anonymised, age representative Norwegian sera (n = 253) collected in 2011 were tested in the haemagglutination inhibition (HI) assay. A HI titre of ≥40 is regarded as seropositive. Influenza A/Indiana/08/2011 (H3N2)v virus, provided by the U.S. Centers for Disease Control and Prevention through the GISRS system, was used as antigen. Data from 1996–1998 was obtained from the Norwegian Annual Influenza Serology Archive.
Results: The overall seroprevalence to A(H3N2)v was 40%. An age-related pattern was seen, which ranged from 0% in children born after 1998 and up to 71% in adults born between 1987 and 1993 (1). A seroprevalence of 40–50% was seen in adults born between 1967 and 1976 and those born in the mid-1950s or earlier. In contrast, low seroprevalence, 14%, was seen in adults born between 1957 and 1966. In 1998 the immunity to the current influenza A(H3N2) virus (A/Nanchang/933/95) was highest, 77%, in people born between 1987 and 1993.
Conclusions: The high overall seroprevalence seen in 2011 suggests that A(H3N2)v has a limited epidemic potential in its current form. The high seroprevalence seen in young adults is consistent with persisting immunity caused by exposure in childhood to antigenically related human viruses during the mid-1990s. This was confirmed by serology data from 1996 to 1998, as A/Nanchang/933/95, a A/Wuhan/359/95-like virus, is antigenically most similar to the A(H3N2)v virus. Children, ≤12 years, are most susceptible to A(H3N2)v, previously not exposed to H3N2 viruses of the mid-1990s. Surprisingly, adults born in 1957–1966 appear to have limited immunity to A(H3N2)v. The differences in immunity to A(H3N2)v between adults, exposed to similar H3N2 antigenic variants albeit at different stages in life, remains elusive.
Reference:
1 Waalen K *et al*. Euro Surveill. 2012;17: pii20170.

P1443
Analysis of CD27/CD27L and CD30/CD30L in T lymphocytes exposed to dengue virus

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Purpose/Objective: To analyze if the co-stimulatory molecules CD27/CD27L and CD30/CD30L are differentially expressed in T Lymphocytes exposed to dengue virus.
Materials and methods: Peripheral blood mononuclear cells were obtained from Buffy coats from healthy donors by differential centrifugation over Fycoll-paque^{TM}PLUS. The T lymphocyte subpopulation was purified by anti-CD3-coated magnetic microbeads and these CD3^{+} T cell were assayed for the expression of CD27/CD27L and CD30/CD30L upon culture with: a) Concanavalina A, b) dengue virus, c) dengue virus plus Concanavalina A and d) medium alone. After incubation for 36 h at 37¡C in 5% CO_2, the expression of the co-stimulatory molecules was analyzed by flow cytometry.
Results: The expression of CD27 and CD30 in Concanavalina A stimulation cells was twice as much as that of un-stimulated cells. Pre-incubation with dengue virus inhibited such a response, no significant differences were observed in the expression of CD27L/CD30L on T lymphocytes cultured under the same conditions.
Conclusions: The CD27/CD27L and CD30/CD30L co-stimulatory molecules are related to the proliferation of T cells. Dengue virus seems to downregulate the expression of these co-stimulatory molecules, partially explaining the abnormal proliferation of T lymphocytes observed *in vitro* assays. Supported by SIP:20120911, and COFAA-IPN.

P1444
Analysis of the essential nature of viral proteins considered to be important target antigens against CMV in candidate vaccine strategies in the guinea pig model

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Purpose/Objective: Development of a HCMV vaccine is a major public health priority. Species specificity precludes the direct study of HCMV in an animal model and pre-clinical study of intervention strategies requires the use of an animal CMV. The guinea pig is the only small animal model for congenital CMV. In HCMV infection, the viral glycoproteins are neutralizing antibody targets and the important protective T cell target antigens are the pp65 and IE1 proteins. Recent analysis of the guinea pig CMV (GPCMV) genome indicates that the virus encodeshomologs to important HCMV genes. We previously demonstrated that GPCMV gB glycoproteinand pp65 protein homologs are antibody and T cell targets respectively. However, little is

known about other candidate vaccine target antigens (glycoproteins and immediate early (IE) proteins). GPCMV transient gene expression and knockout mutagenesis studies were carried out to determine more specific information. Additionally, viral epithelial tropism was investigated in GPCMV to determine if a functional homolog endocytic complex similar to that in clinical strains of HCMV is also present in GPCMV.

Materials and methods: Genes encoding the presumptive HCMV glycoprotein homologs were cloned into expression epitope tagged vectors to analyze protein cellular localization. Individual genes (viral glycoproteins and IE genes) were knocked out in an infectious GPCMV BAC. Pathogenic salivary gland (SG) GPCMV and lab adapted virus were compared in their ability to infect epithelial cells.

Results: Studies indicated that GPCMV encodes functional homologs to HCMV glycoproteins: gB (GP55); gH (GP75); gL (GP115); gO (GP74); gM (GP100); and gN (GP73). Only gO (GP74 gene) was non-essential for virus replication in tissue culture but did modulate the level of gH in mature virions. The nuclear IE proteins (IE1 and IE2) are essential for virus growth. SG virus had epithelial tropism which could lost by passage of this virus on fibroblast cells. Analysis indicated that modified tropism was associated with a homolog to HCMV UL128−131 locus which was rapidly deleted. The homolog UL128 and UL131 proteins interacted with gH and gL in transient expression studies suggesting potential for a endocytic complex similar to that in HCMV (gH/gL/UL128−131).

Conclusions: Overal, l studies indicate that the encoded homolog glycoproteins and IE proteins have similar function to their HCMV counterparts. This initial characterization should aid in the development of candidate CMV vaccines in the guinea pig, including novel strategies against viral epi/endothelial viral tropism which could be potentially important in preventing transplacental infection.

P1446

CD4$^+$ T and B cell antigenic determinants are aligned in secondary dengue infections

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Purpose/Objective: Dengue virus (DENV) is the principal arthropod-borne viral pathogen afflicting human populations. Whilst antibody repertoires to DENV have been linked to protection or enhanced infection, the role of T lymphocytes in these processes remains poorly defined. The objective of this study is to characterize T cell responses in dengue patients, to understand if CD4$^+$ and CD8$^+$ T cell epitopes target distinct viral proteins and whether these proteins are similar to those recognized by B cells.

Materials and methods: T cell responses were assessed in a Singapore cohort of adult patients affected by dengue. T cell epitope reactivities were assesed by screening ex vivo isolated PBMCs with an overlapping peptide library spanning the entire DENV proteome. CD4$^+$ and CD8$^+$ T cell responses were further charaterized by intracellular staining. Linear B cell epitopes were assesed by ELISA with overlapping peptides spanning the entire E protein sequence.

Results: Whereas CD8$^+$ T cells preferentially target non-structural proteins (NS3 and NS5), CD4$^+$ T cell epitopes are skewed towards recognition of viral components that are also targeted by B lymphocytes (E, C and NS1). Within the E protein CD4$^+$ T and B cell epitopes display a significant degree of overlap, suggesting a direct link between antibody-antigen recognition and peptide presentation to CD4$^+$ T cells. Consistently, a large proportion of dengue-specific CD4$^+$ T cells have phenotypic characteristics of follicular helper T cells, providing evidence of their interaction with B cells *in vivo*..

Conclusions: Based on our data we propose that in secondary DENV infections, virus-specific B cells may skew the CD4$^+$ T cell repertoire by selectively supporting CD4$^+$ T cells of matched antigen specificity.

P1447

Characterisation of human natural killer cell responses to dengue-infected monocyte-derived dendritic cells

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Purpose/Objective: Natural Killer (NK) cells are required in innate responses during early viral infections. However, the factors that regulate the response of primary human NK cells to primary human cells infected with dengue virus (DV) remain poorly characterised.

Materials and methods: In this study, we demonstrate the responses of naïve human NK cells co-cultured with DV-infected monocyte-derived dendritic cells (DCs) using flow cytometry and BATDA cytotoxicity assays. Blocking/neutralizing antibodies were used to determine factors that triggered NK cell responses. ELISAs were employed to detect cytokines produced by DV-infected DCs.

Results: NK cells were found to produce IFN-γ, upregulate cell surface CD107a, and lyse DV-infected DCs. Type I interferons and TNF-α produced by the DV-infected DCs were crucial for this IFN-γ response, since blocking IFNAR combined with neutralization of TNF-α completely abolished the IFN-γ response in these co-cultures. Cytokines previously reported to play important roles in NK cell activation, e.g. IL-12, IL-15, IL-18 and IL-27, were not produced in significant amounts by DV-infected DCs. Transwell experiments suggested that membrane-bound ligands were also important for NK cell recognition of DV-infected DCs. However, we failed to detect the upregulation of ligands for NKG2D or DNAM-1 on DV-infected DCs as well as for ligands for the NK cell natural cytotoxicity receptors.

Conclusions: In summary, our results have uncovered a previously unappreciated role for the synergy between Type I interferons and TNF-α in triggering the anti-dengue defence of naïve human NK cells.

P1448

CMV infection in young individuals associates to CD8$^+$ T cells polyfunctionality

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Purpose/Objective: When naïve T cells are activated they become effector cells with different cytokine production patterns. Thus, different cytokine producer T cell subsets have been defined depending on the type and number of cytokines produced. A single T cell can produce simultaneously multiple cytokines of the same type, being then commonly referred as polyfunctional. Several publications have shown that a higher number of polyfunctional T cells is correlated with a better prognosis during HIV infection and vaccine animal's studies have shown that the quality of the response, i.e. polyfunctionality, is predictive of control of the infection following challenge. Thus, this analysis of the T cell response to vaccination and infection has demonstrated that the efficiency of the response is associated with the

capacity of responding cells to produce several cytokines ('polyfunctionality' as a marker of quality) rather than with the percentage (quantity) of specific T cells.

The aim of this work is to analyze the effect of CMV infection on the polyfunctionality -degranulation (CD107a) and/or cytokines co-production (INFg and TNFa) – of $CD8^+$ T lymphocytes, in a cohort of young healthy donors.

Materials and methods: We studied 20 young healthy donors (18–35 years old), stratified by CMV serostatus. Cells were stimulated for 5 h with SEB (*Staphylococcus enterotoxin* B) or with pp65/IE-1 CMV peptides. Following stimulation, cells were stained and analyzed on a 7 color MACSQuant instrument (Miltenyi Biotech).

Results: Our results showed that, after stimulation with SEB, $CD8^+$ T cells of CMV+ subjects are more polyfuntional (degranutation, IFNg and TNFa production) than $CD8^+$ T cells of CMV- individuals, whose $CD8^+$ T cells are predominantly monofunctional (TNFa or CD107a). We also observed an increase in $CD8^+$ $CD57^+$ T cells in CMV+ individuals when comparing with the CMV-. In addition, after stimulation with CMV peptides (pp65/IE-1), $CD8^+$ T cells exhibit the same pattern of polyfunctionality as when stimulated with SEB, but the differences between CMV+ and CMV- individuals were higher.

Conclusions: CMV infection in young individuals enhances the functionality and therefore the quality of $CD8^+$ T cells. This effect is associated to the increase of $CD8^+$ $CD57^+$ T cells in CMV+ young individuals.

P1449

Complex interferon- and immunotherapy in the treatment of immunocompromised children suffering from often and long recurring symptoms of respiratory infections

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Purpose/Objective: Immunocompromised childrenwho suffer from recurrent (from 4–6 to 16–24 episodes a year) acute viral and/or bacterial respiratory tract infections are referred to as 'difficult patients', that is patients with frequent diseases. Earlier we had shown that such states develop on the background of disturbances in the function of the immune system (IS) and interferon (IFN) system (I. Nesterova *et al*., 2005).

Materials and methods: We had studied 48 immunocompromised children (both sex, age 5–9 years) who suffer from recurrent (from 4–6 to 16–24 episodes a year) acute viral and/or bacterial respiratory tract infections. An algorithm for integrated investigation of patients included clinical and anamnestic control, microbiological mucous microbiocenosis investigation, PCR, sulfur diagnostics, testing of antiviral immunity (T chain, humoral chain, neutrophlic granulocytes (NG), natural killers (NK) and IFN status.

Results: All patients had disturbance of IFN status (low levels of induced production of IFNα (100% of cases) and IFNγ (50%) and combine immunodeficiencies (91.6% of cases). A program of interferon- and immunotherapy for those patients was developed. It included basic therapy with Viferon (system and local), using differential doses of IFNα2 (from 1 up to 2 Mln IU) with further reduction of the prolonged course dose (2.5–3.5 months). In case of need, targeted immunotherapy was used. Licopid was used to correct the defects in NK system and/or NG; Isoprinosinum was applied to recover the T chain of immunity; IRS-19 was used in patients with disturbed specific humoral immunity. The use of this integratedprogram of interferon- and immunotherapy in 48 immunocompromised children allowed us to achieve a high clinicaleffects: the level of acute viral and/or bacterial respiratory tract infections was decreased in 3.5-fold, the reconstruction of immune system had more than 85% of all of patients.

Conclusions: Created program of combine interferon – and immunotherapy for immunocompromised children who suffer from recurrent acute viral and/or bacterial respiratory tract infectionshad demonstrated high clinical and immunological effects in 87.5% of cases.

P1451

Differential functional ability of T cells in patients with dengue haemorrhagic fever

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Purpose/Objective: The pathogenesis of dengue haemaorrhagic fever (DHF) is poorly understood. It has been shown that there is delayed clearance of the dengue virus (DEN) in patients with DHF. We set out to investigate if impaired DEN specific T cell responses were associated with development of DHF and dengue shock syndrome (DSS).

Materials and methods: *Ex vivo* ELISpot assays were done to determine DEN3 NS3 peptides specific T cell responses in 40 patients with DHF (10 of them had DSS). Other cytokines produced by DENV-NS3 specific T cells were determined by using multiple bead array analysis on ELISpot supernatants. CD107a expression was determined in 11 patients with DHF and in four healthy volunteers with previous asymptomatic dengue infection by intracellular cytokine assays.

Results: Although not statistically significant, IFNγ DENV3 NS3 specific responses were higher in patients who developed DSS (mean 639.3, SD ± 820.1) when compared to patients who did not develop DSS (mean 313, SD ± 513.1). We did not observe significant production of TNFa, IL-10, IL-4, IL-13, IL-17 by DEN3 NS3-specific T cells. Interestingly, PBMC incubated overnight in the absence of any further stimulation produced large amounts of TNFa, IL-10 and also TGF§ (spontaneous cytokine release). Spontaneous release of TNFα from PBMC ELISpot supernatants was significantly higher ($P = 0.009$), in patients with DSS (mean 2193, SD ± 2094 pg/ml) when compared to those who did not develop DSS (mean 721.4, SD ± 1921 pg/ml). Spontaneous release of IL-10 from PBMCs showed a significant and positive correlation with spontaneous release of TNFα from PBMCs (Spearmans $r = 0.88$, $P < 0.0001$).

CD107a expression was not seen in DEN3 NS3 specific CD8+ T cells in 5/11 DHF patients and CD4+ T cells in 8/11 patients. CD107a expression was seen in 0.25–1.2% of CD8+ T cells and 0.53–1.83% of CD4+ T cells in the 4 healthy volunteers with past asymptomatic dengue infection.

Conclusions: Our data suggest that T cells of patients with DHF and DSS produce high levels of IFNγ, which may be associated with disease pathogenesis. However, their T cells appear to have impaired capacity to degranulate in response to dengue virus proteins, which may delay virus clearance and contribute to severe dengue.

P1452

Do Crimean Congo hemorrhagic fever virus infected human monocyte-derived dendritic cells affect epithelial cell permeability?

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Purpose/Objective: Crimean Congo Hemorrhagic Fever virus (CCHFV) is an arthropod-borne pathogen that in humans causes a

severe disease, Crimean Congo hemorrhagic Fever (CCHF) characterized by hemorrhage and vascular leakage and with high case fatality rate. The mechanisms determining the pathogenicity of this virus is however largely unknown.

We have previously shown that CCHFV entry and release occur basolaterally and that infection does not affect permeability of polarized MDCK-1 cells. We have also shown that CCHFV can infect PBMCs resulting in increased release of Il-,6 Il-10 and TNF-alfa and that infection activates endothelial cells resulting in release of higher levels of IL-6 and IL-8. In this study, we have been interested to set up an *in vitro* model system to study the effect of proinflammatory factors on the tight and adherens junctions during CCHFV infection.

Materials and methods: Human epithelial cells, Caco2 cells were grown on 3.0 μm transwell membranes and resistance measurements were made to ensure confluence before infection. We also isolated human monocyte-derived dendritic cells (DCs) from buffy coats and checked for purity by CD14-positive FACS. Uninfected DCs or supernatant from uninfected DCs were added to the Caco2 model either apically or basolaterally and the resistance was measured.

Results: Our preliminary data demonstrate that Caco2 cells can be infected with CCHFV. Furthermore we found that CCHFV infection *per se* does not have an effect on the permeability of the cell layer.

Conclusions: We have succeeded to set up an *in vitro* model system for studying the role of proinflammatory factors during CCHFV infection and are currently investigating the effect that infected DCs and their supernatant could have on tight and adherens junctions.

P1453

Does hepatitis C virus regulate FcgammaRIIIa expression on NK cells?

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Purpose/Objective: FcgammaRIIIa (CD16) is an activating receptor playing an important role in NK cell function. Previous studies showed that CD16 is down-regulated in HIV-infected macrophages and on NK cells of patients with chronic hepatitis B virus infection, suggesting that it may be involved in the pathogenesis of several chronic viral infections. The aim of the present study was to evaluate CD16 expression on NK cells during chronic hepatitis C virus (HCV) to better understand the possible relationship between virus persistence and the modulation of the FcgammaR IIIa molecule.

Materials and methods: Surface CD16 expression and mRNA levels were examined in NK cells from patients with chronic HCV infection and healthy donors by flow cytometry and by quantitative real-time RT- PCR, respectively. IFNg and TNFa production was analysed by intracellular staining of NK cells after specific stimulation with anti-CD16 antibody. To determine the effect of HCV on CD16 expression, PBMC were exposed to culture-derived cell-free virus or HCV-infected target cells or recombinant HCV proteins.

Results: Expression of CD16 was significantly lower on NK cells from HCV-infected patients compared with healthy donors ($P < 0.0001$). The finding of reduced expression was also supported by lower CD16 mRNA levels in HCV+ patients versus healthy subjects ($P = 0.005$). However, cytokine production mediated by CD16 stimulation was comparable. Following exposure to cell-free HCV, NK cells from control subjects down-regulated CD16, which was confirmed when co-cultured with HCV-infected compared with uninfected Huh 7.5 target cells. Exposure of PBMC to recombinant HCV E2 envelope glycoprotein down-regulated CD16 on NK cells, whereas this effect was not observed with HCV NS3 protease or HIV gp120.

Conclusions: The data suggest that HCV modulates CD16 expression on NK cells. Since CD16 is an important NK cell activating receptor we surmise that regulation of its expression may influence innate immune responses in chronic HCV infection.

P1455

Gammaherpesviruses fail to stimulate IFN responses but induce inflammatory cytokines by a STING-independent pathway

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Purpose/Objective: Herpesviruses are important human pathogenic DNA viruses divided into three subgroups: α-, β-, and γ-herpesviruses. Recent results have indicated that the innate immune response plays an important role in control of α- and β-herpesvirus infections, and that viral DNA is a potent inducer of innate immune responses. Despite the similarities in the family of herpesviruses different DNA receptors may be expected to regulate innate immune responses. Different contribution of receptors to viral recognition, leads to differential regulation of signaling components (e.g. STING a common adaptor protein involved in signaling from most DNA receptors), and therefore diverse cytokine expression. In this project we have investigated the role of DNA-receptors in the innate immune response during an infection with γ-herpesvirus.

Materials and methods: Murine γ-herpesvirus, MHV68, was used to infect cell culture systems and primary macrophages and dendritic cells from wildtype and relevant KO-mice (e.g. STING). Innate immune responses were evaluated by measuring cytokine and type I interferon using RT-PCR, IFN bioassay, and ELISA, accompanied by cellular immunohistochemical staining.

Results: After infection of dendritic cells and macrophages with α-, β-, and γ-herpesviruses we found that the a-herpesvirus herpes simplex virus type 1 and murine cytomegalovirus induced type I IFN and the IFN-stimulated gene CXCL10 and this was dependent on STING, indicating a role for cytosolic DNA sensors in this response. By contract, MHV68 failed to induce IFN responses, even at very high doses of infection. When examining for induction of inflammatory cytokines, we observed that all families of herpesviruses were able to induce such a response, although at different magnitude. Importantly, the MHV68-induced production of TNF-α and IL-6 was not dependent on STING.

Conclusions: The g-herpesvirus MHV68 fails to induce IFN responses in macrophages and dendritic cells, which is in sharp contrast to the STING-dependent induction of type I IFN by a- and b-herpesviruses. Moreover, MHV68 induces inflammatory cytokines in a STING-independent manner, suggesting a role for Toll-like receptors or a novel pathway.

P1456

Genomic and epidemiological dynamics of human metapneumovirus

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Purpose/Objective: Human metapneumovirus (hMPV) is a leading cause of acute respiratory tract infection in young children, with a spectrum of disease similar to that of respiratory syncytial virus (hRSV). The main objective of this study was to identify circulating hMPV lineages and associated sublineages within the local Norwich community (UK) in order to determine the geographical and temporal distribution pattern of hMPV infection within this region.

Materials and methods: The target population included children <18 years attending the Norfolk and Norwich University Hospital with

symptoms of acute respiratory tract infection between October 2005 and December 2008. Viral nucleic acid extraction from nasopharyngeal aspirate samples was performed using the COBAS AmpliPrep total nucleic acid isolation kit and analyser (Roche Diagnostics Ltd) and analysis of the highly conserved fusion (F) gene and the polymorphic glycoprotein (G) gene regions of hMPV was carried out by RT-PCR using primers specifically designed to anneal to the F and G genes. The molecular epidemiology and genetic analysis of hMPV genes was performed using the bioinformatic and molecular biology Geneious Pro™ software.

Results: The findings presented in this study suggest that hMPV exhibits local and global circulation of two distinct genetic lineages, A and B, with switching of the predominant circulating sublineage every 1–3 years. Additionally, phylogenetic data analysis of partial G gene sequences revealed a unique strain or closely related strains within sublineage A2a circulating within the locality of Norwich, which was responsible for large outbreak of infection within the paediatric cohort in 2004/2005, indicating that local factors, such as population patterns of strain-specific immunity, may influence the dynamics of hMPV circulation in different geographical populations.

Conclusions: Phylogenetic analysis of hMPV glycoprotein and fusion gene sequences revealed the frequent displacement of the predominant circulating hMPV sublineage. Additionally, a unique strain was shown to circulate within the paediatric cohort within Norwich, which indicates that local factors may influence hMPV circulation. The high degree of polymorphism observed in the glycoprotein gene has been associated with ability of hMPV to evade recognition by the immune system.

P1457
Genotype cross-reactive antibody responses to hepatitis C virus

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Purpose/Objective: Hepatitis C virus (HCV) is a blood borne pathogen which is responsible for life long, chronic infection of the liver. The humoral immune response to the virus has not been as intensively studied as the cellular response, in part, due to the largely conformational nature of antibody recognition. Here cell culture passaged HCV is used to study the antibody response. The aim of this study was to assess different functions of the humoral response through cross-reactivity for genotype 2 HCV.

Materials and methods: Antigen from cell culture passaged HCV (genotype 2a, JFH-1a strain) was produced by lysis of infected human hepatoma Huh7 cells for ELISA, ELISPOT and western blot analysis. Memory B cells were quantiated following stimulation with the TLR7/8 agonist, R848, and IL-2 for 5 days. Neutralising titres were determined by microassay using HCV infection of Huh7 cells.

Results: Reactivity to JFH-1 virus was found by ELISA for 98% of approximately 100 chronic hepatitis C patients tested prior to treatment. Neutralisation assay revealed that sera from patients of all 4 genotypes inhibited JFH-1 infection strongly. Titres remained high following treatment with interferon and ribavirin. All patients, representing genotypes 1, 2, 3 and 4, tested by western blot recognised HCV core antigen and the majority also recognised the alternate reading frame of core.

Conclusions: Genotype cross-reactive antibody for HCV is common among genotype 1, 2, 3 and 4 HCV patients and core antigenis the most frequent source of cross-recognition. The JFH-1 strain of HCV passaged in human cells can be used for analysis of the specificty, memory and functions of the humoral response to HCV.

P1459
Hantavirus-infection confers resistance to cytotoxic lymphocyte-mediated apoptosis

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Purpose/Objective: Hantaviruses cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardio-pulmonary syndrome (HCPS). Endothelial cells are the main targets for hantaviruses, and increased vascular permeability is a hallmark of HFRS and HCPS. An intriguing observation in patients with HFRS and HCPS is that the virus-infection leads to strong activation of CD8 T cells and NK cells but no obvious endothelial cell destruction. Here, we provide an explanation for this dichotomy by showing that hantavirus-infected endothelial cells are protected from cytotoxic lymphocyte-mediated induction of apoptosis.

Materials and methods: HUVECs were infected or left uninfected and co-cultured with Il-2 activated NK cells. Cell death was measured with FACS (Dead cell marker) or immuno-fluorescence (TUNEL). Cleavage of viral protein was detected by western blot. Granzyme B and caspase 3 activity were measured with specific colorimetric assay.

Results: Our study show that hantavirus-infected endothelial cells are protected from cytotoxic lymphocyte-mediated induction of apoptosis. When dissecting potential mechanisms behind this phenomenon, we discovered that the hantavirus nucleocapsid protein inhibits the enzymatic activity of both granzyme B and caspase 3.

Conclusions: Thus, hantaviruses block cytotoxic granule-mediated apoptosis thereby protecting infected cells from cytotoxic lymphocytes.

P1460
Heligmosomoides polygyrus co-infection reduces lung immune cell responses to respiratory syncytial virus

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Purpose/Objective: Respiratory syncytial virus (RSV) bronchiolitis in infancy may involve Th2 responses and is associated with an increased risk of asthma development. Helminth infection inversely correlates with atopy and allergy in developing countries and can inhibit allergic airway inflammation in animal models, suggesting that they can suppress pathological Th2 responses. Here, we investigated whether a Th2-inducing helminth infection can alter the immune responses to RSV infection.

Materials and methods: Female BALB/c mice (6–8 weeks old) were gavaged orally with 200 L3 *Heligmosomoides polygyrus* larvae or PBS 10 days prior to intranasal infection with RSV, or UV-inactivated RSV.

Results: Compared with RSV infection alone, co-infection with *H. polygyrus* significantly reduced the total lung cell numbers on day 8 post infection (p.i.). Using flow cytometry, we found that the increases in both $CD4^+$ and $CD8^+$ T cell, B cell and conventional dendritic cell (cDC) numbers, all seen in RSV infection, were significantly reduced in co-infection, as were numbers of $CD8^+$ $CD11c^+$ activated cytotoxic T cells. Cytokine analysis of lung homogenates on day 8 p.i. did not

reveal changes in the Th1 type cytokines, but did show increased Th2 type cytokine responses to RSV in co-infection, with significant differences for IL-4.

During early RSV infection (day 2 p.i.) numbers of natural killer (NK) cells increase and are thought to be important in limiting viral titres. Following co-infection we did not find any increase in lung NK cell numbers. However, on day 2 p.i. we did find elevated numbers of basophils in lungs from co-infected mice which were not detected in RSV infected controls. In addition, co-infected mice had significantly lower levels of TH1 type cytokine responses, including IFN-γ, TNF-α and IL-6, during the early stages of RSV infection.

Conclusions: Co-infection with *H. polygyrus* reduces a broad range of immune cell responses to RSV infection, but induces lung basophils and results in decreased early TH1 type cytokines responses to RSV and increased Th2 type cytokines later in infection. Thus, helminth infection can alter immune responses to RSV infection and could possibly result in reduced inflammation and disease. The mechanism behind interactions of helminths with RSV and its consequences require further investigation.

P1461
HLA-G effects on Crimean Congo hemoragic fever

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Purpose/Objective: Objective: Innate immunity generates the first defencive step during viral infections. Viruses try to reduce innate immunity to enhance their virulance. Crimean-Congo Hemorrhagic Fever virus (CCHFV), a member of the genus Nairovirus, family Bunyaviridae, causes to a severe hemorrhagic fever and may be fatal in humans. Progression is very fast and after an incubation period of 1–3 days severe hemorrhagic symptoms may occur on 3th or 5th day. It has been previously shown that many diseases are releated to human leukocyte antigen (HLA) molecules important in presenting of antigens. HLA-G, a member of HLA class I molecules is different from other members of this class and named also as non-classical class Ib molecule.

Purpose: In this study we planned to investigate HLA-G effect on CCHF.

Materials and methods: We measure the plasma levels of HLA-G, serum levels of IL-6, IL-10, TGF-beta by ELISA and proportion of, $CD16^+$, $CD56^+$, $CD14^+$, $CD4^+$ 25^+ and HLA-G+ cells by flow cytometry of 60 CCHF patients and 60 healthy volunteers. Results are analysed statistically via Mann–Whitney *U*-test *ve Student's T-test*.

Results: Our resuls showed that serum IL-10 levels and HLA-G expression on monocytes, NK and Treg cells are increased in patients group. During the infection HLA-G expression on monocytes, NK and Treg cells were significantly higher than recovery patients. In mortal group, HLA-G expression on monocytes, NK and Treg cells were and serum IL-6 and IL-10 levels were increased.

Conclusions: In conclusion, CCHF virus infection induce HLA-G expression on cell surface and HLA-G plasma levels. Incresed levels of HLA-G expression and plasma levels could exhibit strong immunosupressive activities. Our data showed that, fallowed HLA-G expression on cell surface may be a prognostic and predictif factor for CCHF virus infection. The importance of these results are the fist report to demostrate clinical aspects of HLA-G expression and serum HLA-G levels in CCHF virus infection.

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P1462
Human oncogenic retrovirus HTLV-1 replication is suppressed by the MHC class II transcriptional activator CIITA via an inhibitory action on the viral transactivator Tax-1

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Purpose/Objective: The aim of the study was to evaluate whether the master regulator of MHC class II gene transcription CIITA inhibits Tax-1-mediated activation of viral LTR promoter and thus the replication of Human T-cell Lymphotropic Virus type-1, the causative agent of an aggressive malignancy of $CD4^+$ T lymphocytes.

Materials and methods: Inhibition of Tax-1 transcriptional activity was assessed by luciferase gene reporter assay. Sub-cellular localization of proteins was analyzed by immunofluorescence and confocal microscopy analysis. Proteins interaction was assessed by coimmunoprecipitations experiments. HTLV-1 viral expression was evaluated by transfecting the HTLV-1 molecular clone in 293T and U937 cells and measuring p19 production.

Results: CIITA inhibits HTLV-1 replication by blocking the function of the viral transactivator Tax-1 both when exogenously transfected in 293T cells and when endogenously expressed by U937 promonocytic cells. Tax-1 and CIITA physically interact *in vivo* via the first 108 aminoacids of Tax-1 and two CIITA adjacent regions (1–252 and 253–410). Interestingly only CIITA 1–252 mediated Tax-1 inhibition, in agreement with the fact that CIITA residues from positions 64–124 are required to block Tax-1 transactivation. CIITA inhibitory action on Tax-1 correlates with the nuclear localization of CIITA. CIITA severely impairs the physical and functional interaction of Tax-1 with the cellular co-activators PCAF, CREB and ATF1, which are required for the optimal activation of HTLV-1 promoter. Accordingly, the overexpression of PCAF, CREB and ATF1 restore Tax-1-dependent transactivation of the viral LTR promoter inhibited by CIITA.

Conclusions: These results show that CIITA acts as a physiological restriction factor against HTLV-1 by blocking virus replication and spreading. Since CIITA exerts a similar action on HIV-1 and HTLV-2 replication, these results clearly indicate that CIITA has evolved encompassing a dual function: regulator of adaptive immunity and retrovirus restriction factor in intrinsic immunity.

P1463
Identification of an early CXCR3-dependent antiviral mechanism in the female genital tract working prior to the action of interferons

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Purpose/Objective: Recognition of pathogens and a subsequent immune response is important for defense against infections. This recognition is mediated by pattern recognition receptors (PRRs) which recognize conserved pathogen-associated molecular pattern. Vaginal HSV-2 infection of mice results in an immediate release of cytokines which initiate and direct the subsequent immune response. The aim of this project is to describe and characterize the early innate immune response at mucosal surfaces elicited by vaginal HSV-2 infection. To do this we investigated whether vaginal epithelial cells, which are the first cells to come in contact with HSV-2, can recognize HSV-2 and elicit an immune response by secretion of cytokines. In addition we wanted to identify the PRR responsible for the early recognition of HSV-2 in vaginal epithelial cells. Finally we wanted to examine which immune effector cells respond to the cytokines released by the epithelial cells.

Materials and methods: Mice are infected vaginally with HSV-2. The mice were examined post infection (p.i.) and scored for disease development. Viral load measurements and cytokines concentration were determined in vaginal washes day 1, 2 and 3 p.i. The presence, recruitment and activation state of immune effector cells were examined in isolated vaginas by flow and IHC. PRRs expression in the vagina is investigated by qPCR.
Results: The expression kinetics of several different cytokines in response to HSV-2 infection where investigated and the cytokines CXCL9 and 10 were the only ones induced already within 24 h p.i. This was in contrast to the interferons which were not induced until day 2 p.i. The CXCL9/10 receptor KO mice ($CXCR3^{-/-}$ mice) had elevated virus titer day 1 p.i. and exhibit pronounced signs of disease compared to WT mice. Furthermore there seemed to be a disregulated recruitment of effector cells in $CXCR3^{-/-}$ mice in response to HSV-2 infection.

In uninfected vaginas specific cytosolic DNA sensor was highly expressed.
Conclusions: Our data indicate the existence of a CXCR3 receptor dependent antiviral mechanism working prior to the induction of interferons. Epithelial cells of the vagina recognize HSV-2 and subsequently release CXCL9 and 10. The PRR expression studies indicates DAI as candidate for HSV-2 sensing in vaginal epithelial cells.

P1464
IL-21 has anti-viral effect in innate immunity against vaginal HSV-2 infection in mice

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Purpose/Objective: $CD4^+$ T cells produce IL-21 in response to infection with HSV-2 in mice and IL-21 has anti-viral capacities in the adaptive immune response to chronic viral infection. Little attention has been given the potential role IL-21 could play in innate immunity. This is despite the fact that cells from the innate immune system like NK cells and macrophages readily express the IL-21R and respond to IL-21 treatment with increased effector functions.
Materials and methods: In this study we investigated the role of IL-21 in innate immunity to virus infections using a murine model for vaginal HSV-2 infection in C57BL6 WT and IL-21R KO mice. Mice were infected intra vaginally with HSV-2 and scored for disease progression and death. Viral titers and type 1 IFN levels in vaginal fluids were measured on day 1–3 p.i. In separate experiments WT mice were treated with murine recombinant IL-21 (mIL-21). In other experiments vaginas were harvested on day 1–3 p.i. and RNA was extracted or cells were sorted by FACS in $CD45^+$ and $CD45^-$ fractions for RNA extraction or culturing. mIL-21 and IL21R KO mice were a kind gift from Pfizer.
Results: Here we show that IL-21R expression was increased at the site of infection on day 1–3 p.i. in WT mice. This was due to an increased IL-21R expression in leukocytes ($CD45^+$ cells). IL-21R KO mice had a higher disease score and higher viral titers in vaginal fluids compared to WT. Moreover mice treated with mIL-21 had a significantly lower disease score and showed increased survival compared to infected untreated controls. mIL-21 treated mice also had significantly lower viral titers in vaginal fluids.
Conclusions: We conclude that IL-21 plays a role in innate immunity to HSV-2 infection in the murine vagina. In conclusion our data suggest a protective role for IL-21 in innate immunity against virus.

P1465
Immunophenotyping of mice after influenza A virus infection

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Purpose/Objective: Different inbred mouse strains exhibit striking differences in susceptibility to influenza A virus (IAV) infections. To phenotyping of infected mice we established peripheral blood count analyses and immunophenotyping of the lungs in addition to the commonly used monitoring of weight loss and survival.
Materials and methods: Two inbred mouse strains (DBA/2J and C57BL/6J) were infected with three different influenza A virus H1N1 variants (PR8M, PR8F and hvPR8). The blood analyses were performed with a veterinary hematology system (VetScan). For a more detailed analysis we investigated infected lungs using flow cytometry to study the immune cell populations recruited to the site of infection.
Results: We found significant differences in the relative distribution of the main immune cell populations in peripheral blood already on day two post infection. Susceptible mouse strains exhibited strong lymphocyte decreases, moderate increases of monocytes and very strong augmentation of granulocytes after infection. In comparison, the hemogram of infected resistant mice changed to a much lesser extent. C57BL/6J mice displayed leukocytosis when they survived an influenza A infection and leukopenia if a lethal outcome was observed. We could notice that blood parameters measured early after infection correlate well with the clinical course and outcome of influenza A virus infection in mice. By comparing the lungs of infected DBA/2J and C57BL/6J mice, we observed a high amount of phagocytes in the susceptible mouse strain on day three post infection whereas resistant C57BL/6J mice revealed an augmentation of MHCII expressing cells within the lungs.
Conclusions: In summary we can conclude that the pathogenicity of influenza A infection, depending on genetic susceptibility or virulence of the pathogen, is well reflected in the peripheral blood count. Thus, this method represents a much more sensitive measure compared to weight loss, and it is also predictive for the outcome of the infection. The analysis of immune cell infiltrates in the lung tissue revealed clear differences between susceptible DBA2/J and resistant C57BL/6J mice. To further understand the biological significance of these findings immune cell infiltrates will have to be investigated in more detail and are subject of ongoing research.

P1466
Impact of CMV infection on human dendritic cell TLR expression and function

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Purpose/Objective: A latent CMV infection in humans is associated with accumulations of late-stage differentiated peripheral T-cells and poor responsiveness to influenza vaccination in the elderly. As monocytes and dendritic cells (DCs) serve as a reservoirfor latent CMV, and considering the important role of DCs in T-cell activation, we analyzed the impact of a latent CMV

infection on human dendritic cell TLR expression and function.
Materials and methods: We analyzed the impact of a latent CMV infection on the distribution of myeloid (mDC, Lineage-, HLA-DRhi, CD11c+, CD123-) and plasmacytoid (pDC, Lineage-, HLA-DRhi, CD11c-, CD123+) DCs in peripheral blood and the expression level of Toll-like receptors (TLR-2, -4 and -7, determined by mean fluores-

cence intensity) on circulating monocytes ($CD14^+$), as well as mDCs and pDCs in middle-aged healthy individuals ($n = 20$). The responsiveness of immature monocyte-derived DCs to stimulation through TLR-2/6 and TLR-4 was also analyzed in 19 middle-aged healthy donors.

Results: Our results demonstrate a significantly higher expression level ($P = 0.021$) of TLR-2 on monocytes in CMV-seropositive individuals. However, stratifying the donors according to gender revealed an impact of CMV-seropositivity only in men. We also detected a significantly higher expression level of TLR-7 on pDCs ($P = 0.048$) in men with a latent CMV infection. The distribution of different DC subsets, the mDC/pDC ratio and the expression level of TLRs on mDCs, did not differ between CMV-seropositive and *seronegative individuals. However, functional experiments with monocyte-derived DCs revealed a strong trend ($P = 0.05$) towards higher up-regulation of CD86 in CMV-seronegative individuals in response to stimulation through TLR-2/6, but not TLR-4.

Conclusions: These results suggest a potential impact of a latent CMV infection on the capacity of DCs to respond to different TLR-agonists. Thus, CMV-serostatus might have an impact on responsiveness to different adjuvanted vaccines, as well as the functional capacity of monocyte-derived DCs used in different clinical settings, such as cancer immunotherapy.

P1467

Increased pathology during infection with an atypical European porcine Arterivirus correlates with an enhanced adaptive immune response

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Purpose/Objective: The porcine reproductive and respiratory syndrome virus (PRRSV) causes one of the most economically important diseases of swine worldwide. PRRSV replicates primarily in alveolar macrophages, and outside the lung is mainly found in the lymphoid tissues. Respiratory infection of piglets with European strains normally results in subclinical disease and mild pathology. However, recent years have seen the emergence of more pathogenic strains. This study aimed to characterise the immune response to a virulent European PRRSV strain.

Materials and methods: Pigs were inoculated with the prototype European strain, a British field strain or a recently isolated strain from Belarus (SU1-bel). Lung gross pathology was scored and bronchoalveolar lavage fluid (BALF) collected at 3, 7 and 35 days post-infection (dpi). Viral loads were determined by qRT PCR. Immunophenotyping was performed using flow cytometry, and adaptive responses were analysed by IFN-γ ELISpot and PRRSV-specific Ab ELISA.

Results: Clinical signs and gross lung pathology were most severe in the SU1-bel infected pigs with inflammation particularly pronounced at 7 dpi. Viral load in the lung did not correlate with pathology, and was lowest in the SU1-bel pigs at 35 dpi. The SU1-bel pigs mounted a stronger cellular immune response with higher numbers of IFN-γ secreting cells in both the blood and lungs. The humoral response was also greater with earlier detection of PRRSV-specific Ab in the BALF and serum, although these were not neutralising. At day 7, the SU1-bel group showed the greatest influx of both myeloid and lymphoid cells into the lungs, with numbers of CD4 and CD8 T cells and neutrophils all positively correlating with pathology.

Conclusions: This study suggests that the basis of PRRSV pathogenesis is immune-led, rather than a direct effect of the virus, as has been suggested for the related SARs-CoV. Although IFN-γ is anti-viral, and may be a contributing to viral clearance, it is likely that this pro-inflammatory cytokine is exacerbating tissue damage. Work is ongoing to further characterise the cellular immune response in the lung of SU1-bel infected pigs and investigate the innate immune factors, which may contribute to this enhanced adaptive response.

P1469

Interferon- and immunotherapy in immunocompromised patients with chronic recurrent herpes simplex infection

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Purpose/Objective: The treatment of immunocompromised patients with chronic recurrentherpes simplex infection is very actual problem. IFN system is the major part in antiviral immunity provides fast antiviral and protective effects. By numerous authors it was shown that viruses of a herpes simplexI type are capable to undertake countermeasures and to carry out damage IFN system and antiviral mechanisms of immune system, that complicates them elimination and promotes their long persistence.

Materials and methods: Method: We investigated 95 patients at the age from 20 till 55 years, suffering with chronic recurrent herpes simplex type I infection l herpes (HSV1 or HSVII) infection with oral and facial localization. The frequency of acute episodes was 6−14/year. The state of IFN system (induced production of IFN alpha and IFN gamma and the basic mechanisms of antiviral immunity (T lymphocyte's and humoral chains, NK cells, phagocytic cells) were studied.

Results: All patients had combine defects ofIFN alphaand IFN gamma induced production, and some defects of functioning ofimmune systems. Complex program of interferon- and immunotherapy was developed. This program included: (1) carrying out basic system differentiated interferon therapy with application recombination IFNα2 in a complex with antioxidants -viferon, (2) restoration of lymphocyte chain of immune system with using of 10-day izoprinozin course (4), (3) restoration NK, humoral chain, neutrophilic granulocytesand their phagocytic activity by basic and supporting courses oflicopid − drug with multi potential properties. Features of treatment in acute period ofHSV1infection Synthetic antiviral drug famvir was used. The importance of replacement therapy by adequate doses ofviferon at the first stage of treatment was demonstrated. It was shown necessity for the further decrease in doses viferonfor realizationofinterferon correcting and modulating influences inpersons with secondary defects in IFN system. Primary innate defects of IFN system were needed in replacement basic prolonged IFN therapy by viferonduring long time and then in support viferon therapy during life.

Conclusions: Conclusion: It was demonstrated expressive clinical and immunologic efficiencies of the developed program of interferon − and immunotherapy in chronic recurrent HSV1 infection in 82.3% of patients. The frequency of acute episodes ofchronic recurrent herpes simplex type I/IIinfection with oral and facial localization haddecreasedtill2.1 ± 0.05/year ($P \leq 0.001$). IFN and immune system were restored in 82.3% of patients.

P1470

Ligation of mouse hepatitis virus type 3 (MHV3) to TLR-2 decreases interferon type I transcription and favours viral replication by a lipid raft/caveolin dependent mechanism

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Purpose/Objective: Innate immunity is the first line of defense against viral infections and consequently many viruses have developed immune evasion mechanisms, particularly in the liver. Macrophages

possess toll-like receptors (TLRs) at their surface and in endosomes to detect viral infections leading to the induction of antiviral interferons (IFN) α, β and inflammatory cytokines. Recently, surface TLRs, notably TLR-2, have been involved in the recognition of viruses, such as hepatitis B and C, but their role in pathogenesis remains elusive. We have reported that TLR-2/heparin sulfate may act as a receptor for the highly virulent murine hepatitis virus 3 (MHV3), increasing IL-6/TNF-α production. The aim of this work is to verify whether the fixation of MHV3 onTLR-2 modulates the antiviral IFN-β response and/or viral fixation/internalization steps.

Materials and methods: The murine macrophage J774A.1 cell line, permissive to MHV replication, was infected with the highly virulent L2-MHV3 or lesser virulent MHV-A59 variants in the presence or absence of specific inhibitors of various endosomal pathways and levels of TLR-2, IFN-β, viral nucleocapsid and cytokine gene expression were determined by qRT-PCR and/or ELISA tests. The modulating effect of TLR2 in IFN-β production and viral replication was confirmed with TLR-2 and/or CEACAM1a (the MHV receptor) siRNA treated cells.

Results: Our results demonstrate that the MHV3 serotype is significantly more virulent than MHV-A59 in macrophages, as evidenced by higher nucleocapsid expression. However, TLR-2 and IFN-β mRNA levels are lower in cells infected with MHV3. TLR-2 and CEACAM1a siRNA treatments confirm that both receptors are involved in viral load and subsequent IFN-β response. Interestingly, viral load, TLR-2 and IFN-β expression are significantly dependent on the clathrin endosomal pathway for both variants. Indeed, they drastically increase in MHV3 infection when this endosomal pathway is favoured at the expense of the lipid-raft/caveolin mediated pathway.

Conclusions: Taken together, these results suggest that the ligation of MHV3 to TLR-2 enhances viral replication whilst abating the type I IFN response by a still unknown immune evasion mechanism. Furthermore, in stark contrast to MHV-A59, viral load of the MHV3 serotype is remarkably heightened when endosomal entry is shifted to the clathrin-dependent pathway.

P1471

Massive CD8$^+$ T cell activation during acute dengue: role of T cells in dengue pathology?

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Purpose/Objective: Dengue virus (DENV) infections have increased alarmingly over the last decades with an estimate of 50 million people affected per year. DENV is an arthropod-borne flavivirus that exists as four distinct serotypes (Den 1–4). Severe disease has been linked to secondary infections with heterologous serotypes suggesting an important role of pre-existing immunity in dengue pathology. In this study we sought to better understand the role of T cells in mediating protection and/or dengue immuno-pathology. T cell responses have been characterized in a Singapore cohort of adult patients affected by dengue during three time points of disease.

Materials and methods: CD8$^+$ T cells from ex-vivo isolated PBMCs were stained with antibodies against the activation and proliferation markers CD38/HLADR and Ki67/Bcl2 and analysed by flow cytometry. Dengue virus specific T cells were assessed by IFN-γ ELISPOT using a peptide library spanning the entire dengue proteome. Furthermore with the use of HLA tetramers specific for two epitopes derived from the DENV NS3 protein the frequencies, phenotypes and cytokine producing capacities of CD8$^+$ T cells specific were investigated.

Results: We observe that DENV infection induces massive activation and proliferation of CD8$^+$ T cells during the acute phase of infection with up to 70% of cells expressing activation and proliferation markers. In contrast, only up to 1% of circulating CD8$^+$ T cells are specific for DENV as assessed by IFN-γ ELISPOT. Using previously described MHC tetramers for the persistent cytomegalovirus (CMV), we showed that CD8$^+$ T cells specific for CMV also display an activated phenotype, are proliferating and produce IFN-γ during acute dengue. This implies the total activated CD8$^+$ T cells contain populations of T cells specific for unrelated antigens.

Conclusions: The discrepancy between the total CD8$^+$ T cell activation profile and the frequency of circulating DENV specific T cells as well as the activated profile of T cells specific of CMV indicates a bystander activation of unrelated T cells during acute dengue. The factors driving the proliferation of these bystander activated cells and their role in Dengue viral infection are currently under investigation.

P1472

MHC II tetramers visualising CD4$^+$ T cell responses to primary and persistent Epstein-Barr virus (EBV) infection: atypical kinetics of the EBNA1 response

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Purpose/Objective: CD4$^+$ T cells are key orchestrators of virus-induced immune responses, being important for the development and/or maintenance of both humoral and CD8$^+$ T cell-mediated immunity. Yet relatively little is known about the characteristics of virus-specific CD4$^+$ T cell responses at the single cell level in man. Here we use novel MHC II tetramers to carry out the first concerted analysis of the human CD4$^+$ T cell response to primary and persistent infection with the gamma-herpesvirus EBV at the single cell level.

Materials and methods: Nine MHC II: epitope peptide tetramers representing a range of EBV latent and lytic cycle proteins were used in multicolour flow cytometry, along with a panel of cell surface phenotyping antibodies, to visualise the evolution of CD4$^+$ T cell responses to EBV. Our donor cohort included 21 acute infectious mononucleosis (IM) donors undergoing primary infection, of which 11 gave further donations through to convalescence, and 10 EBV seropositive donors with no history of IM.

Results: In Acute IM, MHC II tetramer-positive CD4$^+$ T cells specific for the latent antigen EBNA2 and lytic cycle antigens were expanded to frequencies that reached 10-fold above those seen during viral persistence. The diverse range of epitopes targeted by the highly activated CD4$^+$ T cell response led to skewing of the phenotype of the overall CD4$^+$ population. During the course of IM, responses were rapidly culled to values typical of life-long virus carriage, where most tetramer-staining cells display conventional memory markers but some, unexpectedly, revert to a naïve-like phenotype. Interestingly, CD4$^+$ T cell responses to EBNA1 epitopes were delayed in primary infection, appearing in the blood 2–13 months post IM. This delay may reflect differences we have detected in the processing of the different viral antigens *in vitro*.

Conclusions: Primary EBV infection drives expansion of a broadly targeted, highly activated CD4$^+$ T cell response that quickly reverts to healthy virus carrier state. There is a delay in development of a CD4$^+$ response to EBNA1 which parallels the well-documented delay in antibody response to the same protein. We suggest, from an *in vitro* system, that this may be explained by antigen supply.

P1473

Modulation of influenza virus mediated immunopathology; the role for SOCS proteins

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Purpose/Objective: Cytokine storms are aggressive host cytokine responses causing excessive inflammatory damage, characterized by high levels of TNFα and IFNγ. Activation of cytokines is normally tightly controlled to prevent excessive inflammatory damage. Important regulators of cytokines are suppressor of cytokine signaling (SOCS) proteins. We *hypothesize* that influenza virus interferes with SOCS function as a regulator of cytokines, contributing to the generation of cytokine storms.

Materials and methods: BALB/c mice were inoculated intranasally with increasing doses of of A/WSN/33 influenza A (H1N1) virus. Mice were monitored daily for weight loss and clinical signs. Lungs were harvested 2, 4, 6 and 8 days post-infection. The lungs were fixed with 10% formal saline for immunohistochemistry (IHC), or homogenized for assay of virus titres by plaque assay on Madin-Darby canine kidney (MDCK) cells.

Results: We established a model of influenza infection which resulted in different clinical outcomes; BALB/c mice inoculated with 100 plaque forming units (pfu)/mouse of A/WSN/33 influenza A showed mild pathology with no loss of weight. However, mice inoculated with 10 000 pfu/mouse showed 25% weight loss and severe pathology. SOCS1 expression determined by IHC on lung sections 4 days post-infection, when virus titers peak, was below that observed at baseline (day 2), in all infected groups. SOCS1 expression was maximal at day 8 in the 100 pfu/mouse group. In the 10 000 pfu/mouse group, SOCS1 expression remained at baseline levels throughout the time course of infection. Lung titers (4 days post-infection) showed only a twofold difference between the high and low virus dose.

Conclusions: SOCS1 expression appears to be inversely correlated with virus titers, with decreased SOCS1 expression when virus titers are highest. Expression of SOCS1 is highest at day 8 in the 100 pfu/mouse infected mice, which appear to control viral infection and show no clinical symptoms. Whereas, SOCS1 expression is lowest in 10 000 pfu/mouse infected mice, which lost most weight and had severe pathology. There was not a significant difference in the virus load between the high and low virus challenge groups, yet one appears to control inflammation better. Is the difference in the SOCS expression key?

P1474

Orthopoxvirus seroprevalence in cattle and human in rural areas of São Paulo State, Brazil

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Purpose/Objective: Viruses of the genus *Orthopoxvirus* (OPV) are known to present and promote immunological cross reaction; infection or immunization with a virus of the genus confers protective response to the other. *Vaccinia virus* (VACV), a member of this genus was widely used in the production of vaccine against smallpox during the campaign of the World Health Organization (WHO). Since the end of the global program of mass vaccination, when WHO declared the eradication of smallpox in worldwide, VACV outbreaks causing a zoonotic exanthematic disease, characterized by the appearance of cutaneous lesions on udders and teats of cows and hands of milkers has been reported in Brazil, resulting in economic losses through decreased of milk production and humans infections. This study aimed to carry out a serological study in areas with and without history of VACV outbreaks affecting cattle and milkers in S'o Paulo State, Brazil.

Materials and methods: Blood samples were harvested from dairy cows and milkers of 48 farms distributed throughout the counties of Torre de Pedra, Bofete e Anhembi in S'o Paulo State. After centrifugation the serum samples was obtained and submitted to virus neutralization test. Epidemiological investigation was applied to obtain relevant information such as history of outbreaks affecting cattle and humans and age of the milkers.

Results: From 438 cows analyzed, 178 presented antibodies against OPV. Concerning to 58 milkers, 28 were reagents from which six of them have never been vaccinated against smallpox once their age ranged from 13 to 29 years. The others were vaccinated when young during the campaign of eradication to smallpox.

Conclusions: The presence of antibodies agains OPV in milkers never vaccinated against smallpox suggest their exposure to OPV circulating in environment. In fact, the results of epidemiological investigation reported history of lesions on udders and teats of cows and sometimes lesion in milker's hands. In these areas, previous studies identified VACV as zoonotic agent of outbreaks, so the sera positivity of cows and milkers may indicate environment virus maintenance. The positivity of vaccinated persons can be due to previously specific memory cell response stimulated by natural contact during the outbreaks.

P1475

Proliferation of human T lymphocytes is down-regulated by dengue virus

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Purpose/Objective: To analyze some mechanisms involved in the lack of activation and proliferation of T Lymphocytes exposed to dengue virus serotype-2, *in vitro*.

Materials and methods: Peripheral blood mononuclear cells (PBMC) were obtained from Buffy coats from healthy donors. The T lymphocyte subpopulation was purified by anti-CD3-coated magnetic microbeads. T cells were cultured under: (1) Concanavalin A, (2) dengue virus, (3) dengue virus plus Concanavalin A and (4) medium alone. After incubation for 4–16 h at 37¡C in 5% CO_2, T cell were Analyzed for (1) proliferation by using carboxy-fluorescein diacetate, succinimidyl ester (CFSE) and flow cytometry, (2) Expression of CD25, by flow cytometry, (3) Synthesis of IL-2, by ELISA and (4) activation and nuclear translocation of the transcription factors NFAT and NF-kB, by confocal microscopy in whole cells, and by flow cytometry of isolated and labeled nuclei.

Results: The proliferation of T lymphocytes was inhibited in Concanavalin A stimulated cells when pre-incubated with dengue virus. The synthesis of IL-2 was inhibited when these cells were incubated with DENV-2 before addition ofCon A. A reduction in IL-2Ra expression was also observed, by flow cytometry. The nuclear translocation of NF-AT and NF-kB was diminished when Con A-stimulated T cells were previously exposed to DENV-2.

Conclusions: Understanding the role of the immune system in the pathogenesis related to dengue virus will help to improve patients« treatment. Some reports indicate that patients infected with dengue

virus have a dysfunctional proliferation of T lymphocytes. However, studies *in vitro* have not been performed to analyze in more detail the DEN-2-mediated mechanism(s) of inhibition. As shown in this study, inhibition of nuclear translocation of NFAT and NFkB by DEN-2, as well as inhibition of IL-2 synthesis and IL2Ra expression could be accountable for the observed dysfunctional proliferation of T cells. The present work was supported by SIP: 20120911 & COFFA-IPN.

P1476

Respiratory syncytial virus infection augments NOD2 signalling in an IFN dependent manner in human primary cells

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Purpose/Objective: Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infections in infants, with high variability in disease severity. It is unknown if the presence of specific bacterial species in the nasopharynx may affect RSV pathogenesis. We investigated how bacterial components modulate the inflammatory response to RSV infection in human cells.
Materials and methods: Human PBMC from healthy volunteers and Crohn patients homozygous for the 3020insC frameshift mutation (NOD2fs) were stimulated with RSV A2, MDP and specific PRR ligands. Subsequently, cytokines were measured by ELISA and quantitative PCRs were performed.
Results: Pro-inflammatory cytokine production of human PBMC was increased after stimulation with RSV and muramyl dipeptide (MDP), which is recognized by nucleotide-binding oligomerization domain 2 (NOD2). PBMC from Crohn patients homozygous for a mutation to their NOD2 gene did not show a synergistic response to stimulation with RSV and MDP, suggesting that NOD2 is essential for the observed synergy. Further experiments, which aimed at identifying the viral ligand, indicated that viral RNA plays an essential role. Stimulation with RSV or Poly (I:C) induced IFN-β expression, which resulted in an increased expression of the viral receptors TLR3 and RIG-I, as well as an increased NOD2 expression. Our data indicate that IFN-β induction by viral RNA is an essential first step in the increased pro-inflammatory response to MDP.
Conclusions: We hypothesize that the enhanced pro-inflammatory response to bacterial ligands, such as MDP, following RSV infection may be an important factor in determining the outcome of the severity of disease. These data suggest that the composition of an individual's nasopharyngeal flora may help to determine their susceptibility to severe RSV infections.

P1477

Rhinovirus infections and immunisation induce cross-serotype reactive antibodies to VP1

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Purpose/Objective: Rhinoviruses (RVs) are ubiquitous human respiratory viruses, the major cause of common colds, acute exacerbations of asthma and other respiratory diseases. The development of antibodies to RV following primary infection is not well understood and there is currently no RV vaccine available largely because over 100 serotypes of RV exist. As humans are frequently infected with RV and develop both protective and nonprotective antibody responses, we used mouse models of intranasal RV infection and immunisation to determine the induction, magnitude and specificity of antibody responses to RV.
Materials and methods: Mice were intranasally infected with RV or immunised systemically to determine the induction, magnitude and specificity of antibody responses to RV. Antibody responses to whole RV and individual capsid proteins were determined by ELISA, western blot and ELISpot assays. RV neutralisation was also assessed by *in vitro* assay using HeLa cells.
Results: Strong cross-serotype RV-specific IgG responses in serum and bronchoalveolar lavage were induced towards the RV capsid protein VP1. Both serum and mucosal IgA responses were weaker, requiring two infections to generate detectable RV-specific binding. Similarly two or more RV infections were necessary to induce neutralising antibodies. Systemic and intranasal immunisation strategies boosted homotypic as well as inducing cross-serotype neutralising IgG responses.
Conclusions: Our findings demonstrate the utility of the mouse model for investigating anti-RV humoral immunity and for determining the effect of vaccine strategies on the response to RV infection. VP1 based antigens combined with adjuvants have promise as candidate vaccines to boost serotype-specific as well as cross-serotype neutralising antibodies.

P1478

Selected parameters of innate immunity and mortality in rabbits infected with different strains of RHD virus with different biological properties

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Purpose/Objective: The purpose of this study was to record the view of the innate immune responsein rabbits experimentally infected with 25 European strains of the rabbit hemorrhagic disease virus with different biological properties including haemagglutinating (HA+) and non-haemagglutinating (HA-) strains as well as antigenic variants (RHDVa).
Materials and methods: The rabbits were experimentally infected with twenty five European strains of RHDV with different biological properties, such so as HA (+) (Polish: Kr-1, SGM, KGM, MAL, PD, GSK; French: Fr-1, Fr-2; Czech: V-351, V-561, V-562, V-558; Italian: BS89; Hungarian: 1447V/94); non-haemagglutinating (HA-) strains (Polish: BLA; German: Frankfurt, Spanish: Asturias; British: Rainham) and strains with the variable HA (HA+/-) ability (German: Hagenow; Polish: ZD); as well as HA(+) and RHDVastrains (German: Triptis, Hartmansdorf; Italian: Vt97) and HA (-) and RHDVa strains (Italian: Pv-97; French: 9905). After infecting rabbits, in peripheral blood at times 4, 8, 12, 24, 36 and 48, 52, 56, 60, 72, adherence and absorption capacity were marked in such animals and capacity of NBT reduction in PMN cells, as well as stimulation index and granulocyte metabolic activity index, and the test of intracellular leucocyte killing was performer, as well as the percetage ofTc lymphocytes ($CD8^+$) was marked using flow cytometry. At that time the mortality of animals was also recorded.
Results: The study revealed that the image of innate immunity indices and mortality in rabbits studies varied depending on the biological property of the strains analysed.
Conclusions: The immunological difference in the strains analysed and various mortality of animals caused differentiation of three

immunogroupes (immuntypes) and four pathotypes among such strains of the RHD virus.

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P1479

Specific immunoglobulin G subclass spectrum in previously vaccinated and unvaccinated measles convalescents

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Purpose/Objective: Measles outbreaks continue to occur even in highly vaccinated countries. The aim of study was to investigate peculiarities of specific immune responses in vaccinated and unvaccinated measles convalescents.
Materials and methods: Ten adults with natural measles infection at 5th day (Group 1), nine adults with measles at 12th day after onset of rash (Group 2), and 10 measles patients previously vaccinated against measles (Group 3) were investigated. Twelve healthy adults had two-dose measles vaccination at 1 and 6 years old (Group 4), and 17 healthy adults had measles many years ago (Group 5) were examined as a control groups. Specific IgM, IgA, IgG antibodies in serum, and their subclasses and avidity were tested by ELISA.
Results: All unvaccinated measles patients had a high level of measles IgM antibodies (more than 3.0 OD). The patients in Group 3 had negative or slightly positive specific IgM. None of donors in groups 4 and 5 had any measles IgM. Specific IgG were 0.612 IU/ml in Group 1, and 0.957 IU/ml in Group 2. In healthy donors specific IgG were 0.907 IU/ml in Group 4 (two donors had no measles IgG), and 1.928 IU/ml in Group 5. IgG level in Group 3 differed from others, it was 42.145 IU/ml. In Group 1 the predominant subclass was IgG3. It was present in 100% of serum samples and contributed, on average, 79.2% of the total IgG anti-measles response. In Group 2 the predominant subclass was IgG2. It was 51.5% of total IgG anti-measles response. IgG1 was 19.3% and IgG3 − 26.3% of anti-measles IgG. In Group 4 IgG1 was 67.7%, IgG3 was 18.5%, and IgG2 and IgG4 were low. In group 5 the predominant subclasses were IgG2 − 43.4%, and IgG1 − 34.7% of anti-measles IgG. In Group 3 the predominant subclass was IgG1 -94.3% of anti-measles response. High levels of specific IgA antibodies were founded in all groups.
Conclusions: Differences in IgG subclass profile in vaccinated and unvaccinated measles convalescents could explain as differences in primary and secondary antibody response.

P1480

The control of a moderate dose of MCMV and downstream induction of T Cell responses are dependent on either MyD88 or Ly49H signaling pathways

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Purpose/Objective: The control of Murine Cytomegalovirus (MCMV) infection is known to rely on two mechanisms of innate immunity: (1) virus recognition by dendritic cells (DCs) through their endosomal Toll Like Receptors (TLR) 7 and 9, leading to the synthesis of antiviral cytokines via a signaling cascade dependent on the adaptor molecules MyD88 and IRAK4, and (2) recognition and killing of infected cells by Natural Killer (NK) cells expressing adequate activation receptors such as Ly49H or Ly49P. However, the relative importance of these two mechanisms in the control of infection is unclear.
Materials and methods: To understand this issue, we examined resistance to MCMV infection in 4 BALB/c congenic mouse strains deficient for Ly49H, MyD88 or both.
Results: Three days post-infection (pi), only $Ly49H^+$ mice controlled viral replication in the spleen and liver. However, at day 6 pi, both $Ly49H^-$ $MyD88^+$ and $Ly49H^+MyD88^-$ mice, but not double deficient $Ly49H^-$ $MyD88^-$ animals, had also achieved MCMV control. Antibody-mediated NK cell depletion *in vivo* impaired late viral control in $Ly49H^+MyD88^-$ mice but not in $Ly49H^+MyD88^+$ mice. At moderate doses of viral inoculum, only $Ly49H^-$ $MyD88^-$ mice succumbed to MCMV infection. Hence, both MyD88 and Ly49H were required for control of MCMV early after infection. However, upon moderate dose infection, MyD88 and Ly49H were redundant for late control of MCMV replication and for the promotion of health over disease. MyD88 was instrumental for efficient activation of DCs and NK cells in $Ly49H^-$ but not in $Ly49H^+$ mice. Double deficient $Ly49H^-$ $MyD88^-$ mice failed to mount strong MCMV-specific CD8 T cell responses, which was associated with a major attrition of splenocytes and with extensive tissue damage in spleen and liver as compared to the three other mouse strains studied.
Conclusions: Thus, our results demonstrate that MyD88 is dispensable for the induction of efficient innate and adaptive immune responses against moderate dose MCMV infection in animals which NK cells are able to directly recognize and kill infected cells through the engagement of an activation receptor. This might explain in part why MyD88 and IRAK4 have been reported to be redundant for immunity against viruses in humans. This observation could thus help reconciling the conclusions drawn on antiviral immune defenses from the analysis of animal models as compared to the study of human immunity *in natura*.

P1482

The innate antiviral immunity factor Tetherin does not restrict poxviral replication

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Purpose/Objective: Mammalian cells encode numerous restriction factors to counteract infections with pathogens and inhibit viral replication at cellular levels. In turn, viruses have evolved factors that functionally counteract these intrinsic restrictions. Upon infection, secretion of type I interferons (IFNs) from virus-infected cells is a hallmark of innate antiviral immunity and cells express almost countless interferon-stimulated antiviral genes. One of these interferon-induced cellular restriction factors is Tetherin (also known as BST-2, CD317 or HM1.24), which blocks the release of many enveloped viruses form infected cells. Vaccinia viruses (VACV) encode numerous immune modulators interfering with antiviral host responses. Therefore, it was tempting to study a potential VACV-THN interaction.
Materials and methods: A potential Tetherin (THN)-VACV interaction was analyzed using cell lines either stably expressing a HA-tagged THN and the corresponding cells without THN expression. Cells were infected with VACV and analyzed for viral spread and THN surface expression.

To identify a potential impact of VACV infection on THN cell surface levels, infected cells were stained after infection for THN expression.

To spot possible THN antagonists encoded by VACV that counteract THN by a mechanism distinct from cell surface downregulation we made use of a recombinant MVA, MVA-HERV-K_{gag}, which expresses the human endogenous retrovirus K (HERV-K) Gag/

pol protein. Infection of cells with the recombinant MVA results in the release of HERV-K virus-like particles budding from the MVA-infected cell which are inhibited in their release by THN. Consequently budding of HERV-K particles should be decreased in THN-expressing cells in comparison to THN-negative cells. Potential THN antagonists encoded by MVA should restore the budding phenotype as observed in THN negative cells.

Results: Viral titers were determined at different time points after infection from lysed cells as well as from culture supernatants and were comparable. THN expression levels at the surface of infected cells were not significantly altered. Potential antagonits encoded by VACV would be detected utilising a recombinant MVA expressing HERV-K gag. HERV-K particle release was significantly inhibited in both settings in 293HA-THN cells compared to THN-negative cells.

Conclusions: Our data indicate that poxviruses do not interfere with THN function. THN expression did not inhibit VACV release and replication. VACV infecion did not diminish THN-surface levels or impaired its function on retroviral release. This suggests that THN is unable to restict VACV infections.

P1484

The role of non-neutralizing antibodies in infection with the non-cytopathic lymphocytic choriomeningitis virus

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Purpose/Objective: Persistent infections with non- or minimally cytopathic viruses like HBV, HCV or HIV represent a major health problem world wide. Despite intense research there are hardly any options to treat these diseases. To improve the treatment strategies it is of great importance to gain better knowledge of the effector functions of the immune system that contribute to the elimination of non-cytopathic viruses. Infection of mice with the Lymphocytic Choriomeningitis Virus (LCMV) is a well established murine model for infection with a non-cytopathic virus. We used this model to study the contribution of non-neutralizing antibodies to the anti-LCMV immune response.

Materials and methods: To investigate the contribution of non-neutralizing antibodies to virus control we analysed the effect of passive immune-serum transfer on the anti-LCMV CD8 T cell response in LCMV-infected C57Bl/6 mice.

Results: Passive transfer of LCMV-immune sera or IgG purified from LCMV-immune sera resulted in an accelerated virus clearence in LCMV-infected C57Bl/6 mice. Interestingly, no effect on the virus titer in spleen, liver, lungs, kidney and serum was observed before day 8 post infection. This suggests an indirect effect of these antibodies on virus clearence.

Conclusions: It has been known for decades that cytotoxic CD8 T cells are key players in LCMV elimination. During the acute infection only non-neutralizing LCMV-specific antibodies are detectable whereas neutralizing antibodies against the virus appear only very late, if any at all. Our data suggest that these early non-neutralizing antibodies contribute to the mainly CD8 T cell-mediated immune response to this virus.

P1485

The role of type I interferon in persistent murine norovirus infection

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Purpose/Objective: Persistent viruses have evolved many mechanisms to evade the host immune response including alteration of the host's ability to produce or respond to type I interferon (IFN). Certain strains of murine norovirus (MNV) are able to persist for months following infection, whereas other strains are cleared within days. However, the reasons for these differences and how the host immune system may be involved are not well understood. The importance of type I IFN in the immune response to MNV infection has been demonstrated by findings that type I (along with type II) IFNs inhibit the translation of MNV proteins during infection and that the virus encodes a protein whose functions include delaying the up-regulation of IFN-beta following infection. We have recently characterised a persistent strain of MNV, known as O7, and have investigated whether inhibition of type I IFN induction by infected cells could be a mechanism of immune evasion by persistent MNV strains.

Materials and methods: Immortalised and bone marrow derived macrophages (BMM) and bone marrow derived dendritic cells (BMDC) were infected with different strains of MNV at varying multiplicities of infection and the induction of type I IFN measured by quantitation (ELISA) of the amount of IFN beta in the supernatant at 24 and 48 h post infection. Controls included LPS and poly I:C as well as UV inactivated virus.

Results: We have demonstrated that both immortalised cell lines and primary bone marrow-derived macrophages initiate a significantly reduced type I IFN response on infection by O7 compared with MNV-1. Furthermore, a comparison of the immune response of bone marrow-derived macrophages with bone marrow-derived dendritic cells revealed different response patterns to the virus strains.

Conclusions: We are now investigating whether this difference in cytokine induction is also seen *in vivo* as our results indicate that persistent strains of MNV inhibit induction of type I IFN in certain cell types, which may contribute to the establishment of persistent infection.

P1486

Topical application of imiquimod on distant healthy skin of patients with HSV or HPV infections causes systemic immunomodulation and long-term remission

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Purpose/Objective: Imiquimod, an exogenous ligand of TLR7, can be used as an immune response modifier to generate effective antiviral immune responses to HSV. In this study we evaluated the effect of topical application of imiquimod on distant healthy skin of three patients with HSV and two with HPV infections.

Materials and methods: Three patients with HSV (recurrent herpes labialis) and two with HPV infections (condylomata) and five age and sex-matched healthy volunteers (controls) participated in this study. At the start of an HSV or HPV episode a 62.5 mg quantity of imiquimod (Rx: Aldara; 5% cream, Lavipharm S.A., Athens, Greece) was topically applied under occlusion × 1/day (at bedtime) every day for 3 weeks to a lesion-free abdominal skin area (100 cm^2) of the patient and left in

place for 8 h. The patient was advised to strictly avoid any exposure to sunlight or UV light during the treatment period. Peripheral blood samples were collected prior, during and after cessation of treatment and analyzed by flow cytometry for the estimation of all peripheral lymphocyte populations, including Tregs. Serum cytokines (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-α, IFN-γ) were measured with a cytometric bead array method.

Results: All patients entered long-term remission (>1 year) post-Rx. The controls did not experience any serious side effects during or after Rx. Rx caused proliferation and activation of CD4 and CD8 T-cells, especially the naïve populations, and B1α cells, whereas it caused reduction of CD20 B-cells. $CD4^+$ $CD25^{+/high}$ $FoxP3^+$ Treg numbers (+%) increased within the 1st week post-Rx (from 0.479 ± 0.207% Pre-Rx to 0.696 ± 0.312% 1st week post-Rx, estimated as % of total lymphocytes), and subsequently decreased (0.219 ± 0.141% by 2nd week post-Rx). By the 2nd week, serum IL-6, IFN-α and IFN-γ levels increased (to 47.03 ± 32.52, 19.9 ± 1.07 and 230.3 ± 179.5 pg/ml respectively). Post-Rx (3 months to 1 year), the patients had control levels of all cytokines, increased levels of activated CD8 and CD4 T-cells and B1α-cells, and normal levels of all other lymphocyte populations including Tregs.

Conclusions: Our results indicate that topical application of imiquimod on distant healthy skin can cause systemic immunomodulation and enhance virus specific immune responses. This type of treatment can be an effective therapeutic regimen for HSV and HPV infections.

P1487
Understanding the adaptive immune response to yellow fever vaccine

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Purpose/Objective: The Yellow Fever vaccine, based on a live-attenuated virus (YF-17D), is one of the most effective vaccines ever made. Neutralizing antibodies are thought to be the primary correlate of protection against infection and immunization is known to confer protection in more than 90% of vaccinees. T cells are also thought to have an important role; however, so far only a few studies have investigated primary and memory T cell responses to YF-17D.

While studies on YF-17D virus in humans are limited to the analysis of basic features of the immune response following vaccination, certain aspects of the host response can be studied in much greater detail in a mouse model system.

In this project we use a murine model to investigate the adaptive immune response to YF-17D vaccination and to characterize the role of cellular and humoral immunity in the vaccine−induced protective response.

Materials and methods: In order to investigate cell subsets and effector molecules driving the YF-17D induced immune response, we have used several strains ofknockout mice (ko) in vaccination-challenge experiments; serum transfer and adoptive transfer of splenocytes from YF-17D infected to naïve mice have been performed to study the contribution of cellular and humoral immunity.

Moreover, we have characterized the primary and secondary T-cell responses following vaccination by tetramer staining and intracellular cytokine staining for IFN-γ.

Results: We have observed that while the absence oftype I IFN signaling and of both IFN- γ and perforin have only a very modest impact on the YF-17D induced immune response, both *Rag1* ko mice and MHC class II ko mice fail to develop a protective immune response when immunized prior to challenge. Moreover, in the absence of B cells and antibodies (B cell ko), about 20% of the mice immunized prior to challenge will still develop a protective immune response and survive i.c. challenge, displaying an increased $CD8^+$ T cell response in the spleen compared to surviving wild type mice.

Also, even though transfusion of serum from immune mice to naïve recipients confers 100% protection, adoptive transfer of primed spleenocytes prior challenge also confers protection in about 70%of the animals tested.

Conclusions: Our findings suggest that althoughB cells/antibody are major players in the YF-17D induced protective response in mice, T-cells are also involved.: future experiments will address the specific contribution of$CD4^+CD8^+$ T cells in the vaccine induced protective response in mice.

P1488
Viral load and humoral responses in relation to disease severity in hantavirus infected patients

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Purpose/Objective: Hantaviruses are the causative agents of hemorrhagic fever with renal syndrome (HFRS) in Euroasia and hantavirus cardiopulmonary syndrome in the Americas. The clinical picture and severity of disease varies depending on the particular virus involved. The mechanisms behind the pathogenesis of human hantavirus infections are complex and likely involve both virus and host-mediated mechanisms. It has been suggested that immune responses, including cytokines, cytotoxic T cells and antibodies might have an important role. Thus, the aim of our study was to investigate the viral load and humoral response in HFRS patients. To study possible correlations between viremia, humoral responses and different clinical labparameters to disease severity.

Materials and methods: In total, 105 patients with verified Puumala hantavirus infection were included in this prospective study. Blood samples were collected and clinical symptoms were studied from the acute onset of disease to the convalescent phase. The kinetics of labparameters, viral load and the humoral (IgA, IgM and IgG) response was investigated.

Results: The patients had classical symptoms of acute mild HFRS and 88% of the patients had signs of renal impairment. 1/3 of the patients had low platelets and clinical signs of bleeding manifestations. Fifteen of the 105 patients were classified as having a moderate/severe illness. A correlation was observed between severe illness and low platelets ($P < 0001$), a high creatinine ($P = 0.01$) and a high white blood cell count (WBC) ($P = 0.001$). A high WBC was also associated with renal impairment (high creatinine). Viremia was detected for up to 1 week after symptom debut and the viral load was not associated with severe illness, neither with a high creatinine level. Upon humoral response analysis a low Puumala virus specific IgG response is significantly associated with severe disease ($P = 0.023$), but not when adjusted for age.

Conclusions: During HFRS, levels of viremia did not affect outcome, but the data indicate that low levels of specific IgG in patients could be linked to a more severe clinical outcome in Puumala hantavirus infected patients. A high white blood cell count is linked both to severe disease and to renal impairment. We have previously showed that a there is marked increase of activated CD8 T cells and NK-cells during the acute phase of infection, which could contribute to the severity of disease (Bj*rkstr*m *et al*., JEM 2011; Lindgren *et al*., J Virol 2011). Hence, the level of host defence including neutralizing antibodies and cellular immune responses may play important roles in the pathogenesis of the infection.

Poster Session: Advances in Biologics 1 — Antibodies, Related Molecules and ivlg

P1489

A flow cytometric assay for pharmacodynamic measurements of antibody therapeutics targeting cell surface receptors and its application to a "Site-of-Action" PK/PD model

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Purpose/Objective: (1) To develop flow cytometric pharmacodynamic (PD) assays to assess target expression and coverage by an anti-receptor X therapeutic monoclonal antibody (mAb-A) in pre-clinical species; (2) to utilize these data for qualification of a mechanistic PK/PD model for mAb-A.
Materials and methods: Receptor X numbers/cell and occupancy (RO) by mAb-A or a surrogate antibody in blood and lymphoid tissues from cynomolgus monkeys or mice, respectively, were determined using the Quantibrite System from BD Biosciences and an LSR Fortessa flow cytometer. Target (receptor X) coverage was determined using a combination of antibodies to identify total and free receptor. We first assessed these PD endpoints in a mouse model to qualify predictions of a 'Site-of-Action' PK/PD model and to confirm percent coverage at selected dose levels. We next investigated receptor number and RO in blood and lymphoid cells in cynomolgus monkeys over 2 months following a single 2 mg/kg dose.
Results: In a mouse pharmacology study with surrogate antibody, total receptor X levels on B cells were constant and at the high dose 100% target coverage was achieved, consistent with predictions. Upon a single 2 mg/kg dose of mAb-A to cynomolgus monkeys, receptor X levels in blood were stable and complete RO was observed for the first 19 days, after which RO declined to zero. Loss of RO correlated with the appearance of anti-drug antibodies in these animals. In splenic B cells, significant levels of RO was observed in at study termination (56 days) and total receptor X expression was decreased compared to control animals. This study provides a general workflow for the prediction of target coverage in humans using a site-of-action PK/PD model and confirmation of such predictions from PD data generated through flow cytometric assays.
Conclusions: Flow cytometric assays were developed that characterized total and free receptors/cell from *in vivo* samples in pre-clinical species; similar methodology may be applied to human samples. RO, expression levels, and modulation of expression during disease or upon antibody administration are critical parameters for developing and qualification of the 'Site-of-Action' PK/PD models. This study demonstrates a general workflow for the incorporation of PD measurements into PK/PD models that aid in the prediction of human efficacious doses.

P1490

Abatacept is highly effective in inhibiting T cell priming but fails to induce T cell tolerance after primary antigen encounter

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Purpose/Objective: Absence of co-stimulation in the presence of TCR-stimulation has been linked to the induction of tolerance, deletion or anergy. Abatacept is a CTLA-4-Ig molecule that binds with high affinity to CD80/86 on antigen presenting cells. It modulates CD28-mediated T cell co-stimulation and is currently used to treat rheumatoid arthritis, however it is unknown if its use leads to the development of immunological tolerance. The aim of this study is to gain a greater understanding of the mode of action of Abatacept by investigating *in vivo* its ability to induce antigen-specific immunological tolerance.
Materials and methods: We employed TcR transgenic mice, oral tolerance and adoptive transfer systems to investigate whether abatacept can induce a state of immunological tolerance during primary antigen encounter.
Results: While T cells that have been tolerised by antigen feeding were unable to produce IL-2 after *ex-vivo* restimulation, T cells primed in the presence of abatacept produced copious amounts of this cytokine and resembled naïve T cells. Furthermore, tolerised T cells exhibited a significantly higher proportion of CD25+ $FoxP3^+$ T cells and $CTLA\text{-}4^+$ cells, whereas T cells primed in the presence of abatacept were characterised by the absence of CD25+ $FoxP3^+$, more resembling naïve rather than tolerised T cells. However, abatacept treatment significantly affected T cell activation, as demonstrated by the high proportion of $CD44^{low}CD62L^+$ transgenic T cells in these mice, in contrast to untreated and tolerised T cells that had an antigen-experienced phenotype ($CD44^{high}CD62L^-$). Upon secondary *in vivo* exposure to antigen, abatacept treated T cells expanded to the same degree as naïve or primed T cells, which was significantly greater than expansion observed with tolerised T cells. Furthermore, isolated T cells from abatacept treated mice had a unique transcriptional profile, distinct from naïve, tolerant and primed T cells. These results demonstrate that abatacept treatment significantly modulates antigen specific T cell priming *in vivo*, however does not induce tolerance phenotypically, functionally or transcriptionally.
Conclusions: In this study we demonstrate that abatacept does not induce a state of immunological tolerance after primary antigen encounter, however it significantly affects T cell activation.

P1491

Auto-antibodies in SLE: is antigen microarray the future in autoimmunity diagnosis?

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Purpose/Objective: The successful diagnosis of Systemic Lupus Erythematosus (SLE) is based on clinical presentation as well as multiple autoantibody serological testing; both of which are expensive and time-consuming. This study aimed to optimise and henceforth illustrate the potential use of antigen microarray to detect various autoantibodies for the purpose of screening as well as disease monitoring.
Materials and methods: The procedure was initially optimised by trialling various printing surfaces and slides as well as dilution and blocking buffers. Serum samples were obtained from SLE patients ($n = 19$) and healthy controls ($n = 7$). Followig optimisation serum was analysed by antigen microarray technology to detect 8 known SLE antigens, a Rheumatoid Arthritis (RA) antigen and several positive control antigens.
Results: Optimum results were obtained when antigens were printed in trehalose PBS-Tween onto aminosilane slides (Nexterion®). The lowest background and highest signal-to-noise ratios were obtained when serum was diluted in antibody diluent (Dako). The main finding of this study was that patients had significantly higher autoantibody presence in three of the SLE antigens tested: Nucleosome, Ribonucleoprotein and Ribosomal P ($P < 0.001$, $P < 0.001$, $P < 0.05$), in addition to the RA antigen CCP2 ($P < 0.001$). Other auto-antibodies tested for, such as Anti-dsDNA, showed a non significant higher presence in patients ($P = 0.056$) of which could be due to good control of disease by most patients. Furthermore, antibody levels from patients on treatment correlated with disease improvement.

Conclusions: These results suggest that microarray is an efficient technique to monitor and detect a wide variety of antibodies in multiple patients simultaneously, doing so at a relatively lower cost then current methods. This demonstrates the need for further development of this technology due the potential for providing a means of mass screening, early diagnosis and reliable monitoring of autoimmune disease including, but not limited to, SLE.

P1493

Autoreactive IgG antibodies activity may be suppressed by other immunoglobulins in human serum and colostrum

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Purpose/Objective: The immune system of healthy individuals contains B cells that synthesize IgG antibodies, which recognize a great variety of self antigens. These auto antibodies, can be found in the serum and respectively in the intravenous immunoglobulin preparations, and may interact with antigens that can also be targets of some pathological autoantibodies. Natural IgG autoantibody reactivity can be induces by subjecting the immunoglobulins to extreme environmental conditions (high and low pH values when using affinity elution buffers). This activity is not observed when non-denaturating methods of purification are used.
Materials and methods: We have investigated the *in vitro* interactions between human liver antigens and human serum and colostrum from healthy individuals or pooled human IVIg. Two different methods of IgG purification were performed: immunoaffinity elution (using low pH treatment of antibodies) and soft salt precipitation (no extreme pH value buffers were used). Pure IgA and IgM were obtained from the different fractions of serum and colostral proteins (distribution based on their molecular mass).
Results: Serum or mucosal IgM and IgA isolated by immunoaffinity elution as well as the fraction containing immunoglobulin F (ab')2 fragments possess the ability to inhibit in a dose-dependent manner the interaction of eluted IgG antibodies or pooled IVIg preparation to human liver antigens. These results suggest that the inhibition was due to idiotype-antiidiotype interactions between immunoglobulin molecules before their contact with autoantigens.
Conclusions: The results show that binding of auto-reactive IgG antibodies to self-antigens can be inhibited by serum as well as mucosal IgA and IgM. Our study supports previous findings that IgM has a major role in blocking the IgG autoantibodies. We suggest that normal pooled human IgM and IgA preparations may have a positive immunomodulatory activity in patients with autoimmune diseases, as the control of the auto-reactivity is highly inefficient in those cases.

P1494

Development of a plasma cell specific antibody

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Purpose/Objective: In this project we developed a chimeric antibody for the depletion of multiple myeloma (MM) and autoreactive plasma cells. Multiple myeloma is a malignancy of plasma cells and is classified as a Non-Hodgkin's lymphoma. Although it is the second most common hematological cancer there is no suitable antibody available yet. Today the treatment of choice is the proteasome inhibitor Bortezomib followed by an autologous hematopoietic stem-cell transplantation. Our antibody binds to a plasma cell specific antigen (PCspA) which is not present during other stages of B cell development.
Materials and methods: Heavy and light chain of a monoclonal antibody produced by hybridoma technology were cloned upstream of a human IgG1-heavy and kappa-light chain. The chimeric antibody has been characterized by ELISA, flow cytometry, surface plasmon resonance and *in vitro* cytotoxicity assays.
Results: After validation of the binding specificity and assessment of the K_d we performed *in vitro* cytotoxicity studies. The antibody showed a strong killing of MM cells already after 4 h. Recently, we established a xenograft model of multiple myeloma in mice in which we currently assess the efficiency of antibody-mediated tumor regression. At the same time we are working on the three dimensional structure of the Fab fragment bound to the antigen in order to facilitate the humanization process.
Conclusions: The newly developed chimeric antibody binds with high affinity to its antigen, shows promising characteristics *in vitro* and will be further developed for a possible clinical use.

P1496

Effect of anti-interleukin-2 receptor antibody on human dendritic cell maturation and activation

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Purpose/Objective: Although anti- CD25 (α chain of IL2 receptor) monoclonal antibodies are widely used in transplantation to prevent acute allograft rejection, their effects on human dendritic cells (DC) are poorly understood. A recent study (Wuest *et al.* Nature Medicine 2011) suggests that the main function of CD25 on DC was to transpresent IL2 to activate T lymphocytes. Interestingly, this study could neither found any evidence of signalling in DC by IL2 nor detect the β chain of the receptor (CD122) on the surface of DC. Therefore, we decided to further explore the effects of IL2 in DC.
Materials and methods: Human blood $CD14^+$ cells were selected differentiated into immature DC with IL4 and GM-CSF for 5 days. Maturation was induced by LPS or TNFα in presence or absence of anti-CD25 (Basiliximab, 100 μg/ml) with or without IL2 (100 U/ml) during 48 h. $CD8^+$ T lymphocytes were cultured with allogeneic DC for 5 days after extensive wash. Surface expression of molecules was analyzed by FACS and cytokine secretion was measured by ELISA and FACS. Protein phosphorylation of STAT3, STAT5 and ERK1/2 was detected by Western blot.
Results: First we showed a constitutional expression of β and γ chain of IL2R on DC, while α chain was inducible by LPS and TNFα. Then we found that IL2 induced transcription factor STAT5 phosphorylation in DC. Interestingly, IL2 increased IFNγ synthesis in DC, while anti-CD25 had opposite effects. While these agents don't have any effect on co-stimulatory or migration molecules expression. Finally we showed that IL2 increases the ability of DC to activate $CD8^+$ T cells cytotoxic functions, whereas anti-CD25 decreases these properties.
Conclusions: This study, for the first time, discloses that IL2 induces DC activation through IL2 receptor binding and subsequent intracellular STAT5 phosphorylation. Interestingly, our study suggests a direct effect of monoclonal anti-CD25 on DC that may contribute to their clinical efficacy.

P1498

Fully-human recombinant antibodies are opsonic to genetically diverse Staphylococcus aureus strains

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Purpose/Objective: Anti-surface *Staphylococcus aureus* (SA) antibodies were to be identified and tested functionally for development of a human recombinant polyclonal antibody (HRPA) therapeutic to protect against all SA strains.
Materials and methods: Antibodies to SA were identified based on surface reactivity to formalin-fixed bacteria as measured by Luminex technology. Human anti-SA antibodies reacted to a diverse range of SA strains including USA300 and USA400 strains of Methicillin Resistant SA (MRSA). The antibodies were tested for opsonic activity against several SA strains using two assays * cell-line phagocytosis and whole blood killing. Cell-line assays utilized FITC-labeled SA and opsonization was measured by flow cytometry. Whole blood killing assays (analyzed by viable bacteria counts) were utilized to confirm the cell-line assay results. The use of whole blood demonstrated that our antibody contribution would enhance phagocytosis beyond any background activity from anti-SA antibodies present in human serum. Antibodies were tested both individually and as sets of antibodies.
Results: We identified a number of antibodies able to recognize and opsonize a wide range of SA strains including MW2, LAC, COL and JDK6159 * which represent a diversity of sequence types. The antibodies provided efficacy in these assays to 100 ng/ml or less final depending on antibodies used. The addition of these antibodies was also able to enhance phagocytosis above circulating antibodies found in human serum during whole blood killing assays.
Conclusions: An array of opsonic anti-SA antibodies have been identified and characterized functionally. These new antibodies to naturally antigenic SA molecules allow for strong *in vitro* activity which will contribute to an efficacious HRPA against SA including MRSA strains. By creating HRPAs for therapeutics, it is possible to protect against all SA strains. Targeting multiple epitopes of several different surface molecules will provide protection from drug-resistance common to current antibiotic-based practices.

P1499

Humanisation of DOC2 anti CCR2 antibody; a MAB with therapeutic potential for MS and other autoimmune diseases

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Purpose/Objective: DOC2 a murine anti-CCR2 antibody has been shown to deplete monocytes which express the CCR2 chemokine receptor in both mouse and NHP animal models. Further studies have shown a clinically significant effect in both murine and NHP experimental autoimmune encephalomyelitis (EAE) disease models. We have humanised this monoclonal antibody (MAB) creating a version which can bind both human and NHP CCR2 expressed on monocytes prepared from PBMCs harvested from both species. It has also been demonstrated that this humanised version can induce monocyte depletion in NHP after injection with repeated doses of the MAB. We propose the mechanism for this depletion is by antibody dependent cellular cytotoxicity (ADCC). The main goal of the study is to determine if this humanised MAB can show clinical improvement in the NHP EAE animal model with a view to developing a treatment for MS in humans.
Materials and methods: Humanisaton was carried out by CDR grafting using suitable human donor frameworks for both heavy and light chains of the antibody. The humanised version was tested by flow cytometry using human and NHP PBMCs to demonstrate that the original specificity for the CCR2 target was maintained. NHPs were dosed with the candidate antibody over a 3 week period and flow cytometry monitoring CD14+CD11b+ monocyte depletion was carried out on blood harvested at specific time points throughout the study period. The ADCC assay was carried out by adding the antibody to europium (Eu) labelled CHO cells expressing CCR2 in the presence of NK cells. Target cell lysis was measured by Eu release.
Results: Good binding to both human and NHP CCR2 target on PMBCs was demonstrated by flow cytometry. The antibody also showed a clear ability to induce monocyte depletion in NHPs over the study period. The ADCC assay results confirm that the likely method for monocyte depletion is mediated by this mechanism.
Conclusions: We have established that the humanised anti CCR2 antibody binds to the target and has demonstrated the capacity to induce monocyte depletion in a NHP animal model. Furthermore our ADCC assay results indicate that the monocyte depletion is likely to be mediated by this mechanism. These results indicate that the DOC2 humanised antibody is a good candidate to test in a NHP EAE model with a view to developing a treatment for MS in humans.

P1500

Identification and characterization of a peptide mimetic recognized by disease −associated antiprothrombin antibodies in antiphospholipid syndrome patients

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Purpose/Objective: Antiphospholipid syndrome (APS) is an autoimmune disease characterized by association with recurrent thrombosis, recurrent feral loss, and thrombocytopenia. Antiphospholipid antibodies (aPL) play an important role in thrombocytosis and/or fetal loss related to APS. Prothrombin (PT) is one of the most important targets for the aPL that was considered with prothrombinase activity. To test the feasibility of applying a mimetic specific for a patient-derived monoclonal antiprothrombin [aPT] antibody to study the homologous, disease-associated aPT in APS patients.
Materials and methods: We used the IS6 monoclonal aPT to screen phage-display peptide libraries. In terms of the conserved amino acid sequence in phage peptide selected, peptide and their derivatives were synthesized and analyzed for binding affinity and for their competitive ability to prothrombin from IS6. In addition, the peptides were designed to study IS6-like IgG aPT in serum samples from patients with APS, patients with rheumatoid arthritis (RA), patients with juvenile rheumatoid arthritis (JRA), patients with systemic lupus erythematosus (SLE) but without APS, and normal healthy donors.
Results: After library screening with IS6, four peptides were designed and synthesizes. Analyses of peptides showed that peptide 9099 reacted with IS6 antibody better than the other 3 and inhibited binding of IS6 to prothrombin. The results suggested that peptide 9099 might be a peptide mimetic for the IS6 aPT. ELISA analyses with serum sample indicated that 9099-reactive IgG was present (positivity defined as the mean + 3 SD optical density of the 11 healthy controls) in 27 of 31 APS patients.
Conclusions: The results suggest that the peptide 9099 might be with the potential to develop a specific ELISA distinct disease *associated aPT.

P1501

Impaired langerhans' cell migration in psoriasis: effect of systemic therapies

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Purpose/Objective: Epidermal Langerhans cells (LC) act as sentinels of the immune system: they migrate from the skin to local lymph nodes to present antigen to T cells and are believed to play important roles in the initiation and regulation of immune responses. We have shown previously that *in vivo* LC migration is impaired in the uninvolved skin of patients with early-onset psoriasis (onset <40 years of age). In the current experiments the impact of systemic therapies on LC migration in these patients has been investigated.
Materials and methods: Punch biopsies (6 mm) were taken from non-sun-exposed buttock skin of healthy controls, untreated psoriasis patients or from those patients who had responded well to either T-cell targeted therapies (ciclosporin, methotrexate); anti-cytokine biologics (adalimumab, etanercept, ustekinumab), or fumaderm. Epidermal sheets were prepared, a $t = 0$ control sheet processed for counting, and the remaining sheets floated on medium for 24 h in the presence or absence ofHaCaT (human keratinocyte) cell conditioned medium. Following immunofluorescent staining for CD1a, LC frequencies were assessed.
Results: Similar levels of LC migration to those stimulated *in vivo* by cytokine or allergen (20−30% of cells) were observed in explants excised from healthy controls (mean 19.6 ± 3.1%) in the presence of culture medium alone. However, in biopsies taken from untreated psoriasis patients there was little or no migration under the same conditions (mean 0.9 ± 1.8%). Migration was impaired also in patients who had responded to T-cell targeted therapies (mean -2 ± 4.4%). In contrast in patients who had responded to anti-cytokine biologic agents and fumaderm, migration was restored to similar levels to those recorded for healthy control subjects (mean 17.7 ± 3.9%). We speculated that the lack of LC migration in samples derived from the untreated psoriasis patients was due to deficiencies in the epidermal microenvironment. We therefore cultured epidermal explants from these patients in the presence or absence of supernatants derived from the human keratinocyte cell line HaCaT. There was a significant restoration of LC migration in explants cultured in conditioned medium (mean 23.0 ± 4.7%) compared with control biopsies (mean 2.7 ± 1.3%).
Conclusions: These data indicate that therapies which target key cytokines involved in the pathogenesis of psoriasis can restore LC migration whereas T-cell targeted approaches do not. Further, the restoration of migration by HaCaT conditioned medium suggests that local, keratinocyte-derived factors may be responsible for the lack of LC migration in biopsies derived from untreated psoriasis patients.

P1502

Insertion of artificial cell surface receptors for antigen-specific labelling of hybridoma cells

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Purpose/Objective: M. Listek, B. Micheel, K. Heilmann Insertion of artificial cell surface receptors for antigen-specific labelling of hybridoma cells. Monoclonal antibodies are universal binding molecules and play a central role as powerful tools in biomedical research, therapy and especially in diagnostics. The generation of antibody molecules is generally done by hybridoma technology since published by Kohler and Milstein in 1975. But the identification of antigen-specific antibody-producing hybridoma cells within a heterogeneous cell population is time-consuming and laborious.

To accelerate the screening procedure in order to detect the hybridoma cell of interest we have created a knock in myeloma cell line that express an artificial cell surface receptor.
Materials and methods: To accelerate the screening procedure in order to detect the hybridoma cell of interest we have created a knock in myeloma cell line that express an artificial cell surface receptor.
Results: By bridging the antigen of interest to the artificial system we could clearly show a specific labelling of cells producing antibodies with the desired specificity. These cells were enriched by FACS and cultivated in order to use them for antibody production.
Conclusions: Compared to conventional screening procedures this new approach is able to select antibody-producing cells at an early stage and therefore improve the detection of positive cell clones after fusion.

P1503

Low expression of immunoglobulin G receptor CD16 on mouse NK cells

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Purpose/Objective: Cellular Fcγ-receptors are crucial for mediating the functions of therapeutic antibodies. Antibody dependent cellular cytotoxicity (ADCC) is an important mechanism by which Fcγ-receptor expressing cells of the innate immune system including natural killer (NK) cells can kill antibody-decorated target cells. Whereas the high expression of FcγRIII (CD16) on human NK cells is well documented, data on CD16 expression of NK cell under steady state conditions are barely available. Conclusion from human NK cells are frequently taken as a basis for interpretation of results in mice. We, thus, wanted to measure whether also in mice NK cells reveal prominent expression of CD16.
Materials and methods: Expression of murine CD16 was measured on leukocytes derived from blood, spleen and bone marrow by flow cytometry. Cells were isolated from C57BL/6 and Balb/c mice for comparison. Relative expression levels on murine blood peripheral blood cells types were compared to those on their human counterparts.
Results: Surprisingly, under steady state conditions murine NK cells were among the cell populations with rather low expression levels compared e.g. to inflammatory monocytes, neutrophils and eosinophils, which showed a much more prominent expression both in Balb/c and in C57BL/6 mice. Rather low CD16 expression on NK cells was found in the tested organs/compartments with only slight variations. In general, cells from Balb/c mice revealed significantly higher expression levels than those from C57BL/6 mice. We also compared expression levels on different subpopulations of NK cells, as identified with respect to expression of CD11b and CD27. These subpopulations revealed reproducible but only rather small differences in CD16 expression.
Conclusions: Cell-mediated antibody effector functions like ADCC are often discussed with respect to NK cells as main effectors *a priori* in humans as well as in mice. Since in mice NK cells express much less CD16 than several other populations which, in addition, express a broader repertoire of Fcγ receptors, the former might be overestimated in some models and the latter should also be taken into consideration as potential effector cells.

P1506

Personalised medicine in rheumatoid arthritis: Interleukin-6-receptor polymorphisms rs12083537 and rs2228145 as predictors of responsiveness to tocilizumab

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Purpose/Objective: Immunomodulatory biologics have revolutionized the treatment of rheumatoid arthritis (RA) and other autoimmune diseases. A substantial proportion of patients show no or limited response to these drugs, however, and may suffer irreversible disease progression during treatment. Moreover, the expense of biologics put a tremendous pressure on public health budgets worldwide. Much attention is being paid to development of companion diagnostics that reliably predict patient responsiveness, but so far none have proved clinically useful. Tocilizumab (TCZ; Actemra®), a mAb targeting the IL-6 receptor (IL-6R), has recently been approved in several countries mainly for the treatment of RA and other autoimmune diseases.
Materials and methods: Seventy-eight patients with RA were treated with TCZ. After 3 months, changes in swollen joint count (SJC) and C-reactive protein (CRP) were used as objective measures of any relationship with genotype at the two IL-6R SNPs rs2228145 and rs12083537.
Results: TCZ induced median reductions in SJC and CRP of 76% and 85%, respectively, in 49 patients who were homozygous for the major allele of rs12083537, compared to 100% ($P = 0.06$) and 91% ($P = 0.09$) in 29 patients, who were heterozygous for the SNP. No patients were homozygous for the minor allele of rs12083537. Thirty patients who were homozygous for the major allele of rs2228145 experienced a median reduction in SJC of 66% versus 100% in the group of 37 heterozygotic patients, as well as in 11 patients who were homozygous for the minor allele of rs2228145 ($P = 0.13$). The median reductions in CRP were 85%, 88% and 92%, respectively ($P = 0.68$). Nineteen patients, who were homozygous for the major allele of both SNPs, experienced a median reduction of only 50% in SJC and 69% in CRP, compared to 100% ($P = 0.0047$) and 89% ($P = 0.10$), respectively, in the rest of the patients.
Conclusions: Genotyping of rs12083537 and rs2228145 may prove a useful tool in predicting the clinical response of RA patients to TCZ. Our data suggest that the approximate one fourth of patients who are homozygous for the wild type allele at both loci constitute a group of poor responders.

P1507

Recovery of the T cell receptor repertoire in chronic inflammatory neuropathies with intravenous immunoglobulins: a novel approach to analyze CDR3 spectratyping data

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Purpose/Objective: Chronic inflammatory demyelinating polyneuropathy (CIDP) is an autoimmune disorder of the peripheral nervous system (PNS) with pronounced heterogeneity of disease severity and treatment response. The pathogenic role of T cells remains controversial. Intravenous immunoglobulins (IVIg) are effective in CIDP but their effect on antigen specificity of PNS autoreactive T cells is unknown.
Materials and methods: The T cell receptor (TCR) repertoire of $CD4^+$ and $CD8^+$ T cells in the peripheral blood was analyzed using CDR3 spectratyping. CIDP patients were included without and with IVIg treatment. To simplify the spectratyping analysis, we introduced a new classification system of alterations in the TCR length distribution and correlated this with the statistical values skewness and kurtosis.
Results: While the TCR length distribution of CIDP patients was only moderately altered for most of the Vbeta elements of $CD4^+$ T cells, the $CD8^+$ population displayed extensive oligoclonal expansions in all analyzed 24 Vbeta elements. Treatment with IVIg reduced the oligoclonal expansions within both the $CD4^+$ and $CD8^+$ population. The evaluation of the simplified analysis of spectratyping data correlated with the degree of alterations.
Conclusions: Our data demonstrate that cytotoxic $CD8^+$ T cells exhibit a much broader activation than $CD4^+$ T cells indicating a potentially crucial role of $CD8^+$ T cells in the immunopathogenesis of CIDP. The profound oligoclonal response in T cell activation suggests that multiple peptides may induce and propagate this autoimmune driven disease. The observed reduction of highly activated T cells may contribute to the therapeutic effects of IVIg. The validation of the classification system integrated in a reduction the of time consuming analysis of spectratyping data.

P1508

The creation of immunosensors based on antigen-antibodies against human growth hormone interaction and the application for the analysis in real samples

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Purpose/Objective: The immunosensors combine the selectivity of the binding interactions between immobilized biomolecules and the analyte of interest with the sensitivity and subsequent detection by appropriate detector. The quantification of antibodies in serum samples from patients is essential for evaluation of the immune response and understanding the immunological properties of these proteins. Anti-hGH antibodies at medium-high concentrations inhibited the growth response to administered hGH and seemed to suppress also the effect of endogenous growth hormone. Detection of these antibodies may be useful for the diagnosis of lymphocytic hypophysitis.

The main aim of this research was to investigate simultaneous application of surface plasmon resonance (SPR), electrochemical (EL) and electroassited chemiluminescent (ECL) techniques on the same chip detecting interactions of the specific anti-hGH antibodies with human growth hormone immobilized on a SPR-chip surface. The created immunosensor was applied for the analysis in human serum samples.
Materials and methods: Indirect immunoassay format was chosen for the registration of immune complex formation (hGH/anti-hGH immune complexes using secondary HRP labelled antibodies) using combination of three different physical phenomena and for the formation of new effective platform for immunosensing.
Results: All types of tested analytical signal detection methods (SPR, EL, ECL) showed very good sensitivity for specific anti-hGH antibodies detection. Compared with SPR and ECL methods, EL detection of anti-hGH using pulsed amperometry characterized by the lowest limit of detection (0.027 nM) and the wider linearity range (0.098 * 1.11 nM). Created immunosensor was used for the determination of antibodies against human growth hormone in human serum

samples using the detection of pulsed amperometry. It was found that the recoveries were in the range of 93 * 98%.
Conclusions: This research work demonstrates the successful exploitation of different techniques to detect the specific anti-hGH antibodies using indirect immunoassay format on the same surface. The SPR immunosensor in contract to other used anti-hGH detection methods is suitable for multiple use.

P1509
The IgM CH2 domain as covalently linked homodimerization module for the generation of fusion proteins with dual specificity

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Purpose/Objective: Dimeric assembly of antibody fragments and other therapeutic molecules holds great potential to increase binding and to improve bioactivity. Here, we investigated the use of the IgM heavy chain domain 2 (MHD2) as covalently linked homodimerization module. MHD2 provides the scaffold for bi- or tetravalent molecules with single or dual specificity by fusion of two different scFv fragments to the *N*- and/or *C*-terminus. In addition, the combination of targeting antibody fragments and therapeutic moieties in one molecule results in a tetravalent and bifunctional fusion protein for targeted cancer therapy.
Materials and methods: The scFv fragments directed against EGFR and HER2 were used as targeting components for the generation of both *C*- or *N*-terminally-linked bivalent and monospecific fusion proteins as well as tetravalent, bispecific molecules. Furthermore, a bifunctional construct was generated by the replacement of one of the scFv fragments by a single-chain derivative of tumor necrosis factor (scTNF). All fusion proteins were produced in HEK239T cells. The ability to bind to and mediate killing of target cells was shown in flow cytometry analysis, IL-8 release assays and cytotoxicity assays.
Results: All MHD2 fusion proteins showed selective binding to tumor cells, expressing EGFR or HER2 in FACS analysis. The bispecific scFv-MHD2 fusion proteins exhibited improved binding to cell lines expressing both markers. The antibody-scTNF MHD2 construct exhibited improved TNF activity, also mimicking the membrane-bound form of TNF, as shown by the activation of TNFR2-mediated cell killing. Furthermore, the scFv moiety allowed for an antigen-dependent delivery of TNF to EGFR-positive tumor cells and an improved TNF action on these cells.
Conclusions: Taken together, we established the MHD2 as a versatile module for the generation of bispecific and bifunctional fusion proteins.

P1511
The production of porcine anti-CSF-1R antibodies: reagents to evaluate the effects of CSF-1 on postnatal growth

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Purpose/Objective: Macrophage Colony-Stimulating Factor (CSF-1) is required for the proliferation, differentiation and survival of cells of the mononuclear phagocyte lineage and acts via the CSF-1 receptor (CSF-1R). Mice with a homozygous mutation in CSF-1 are deficient in biologically active CSF-1 (*op/op* mice). Amongst other defects, these mice have low birth weights and are growth retarded compared to litter mate controls. Injecting *op/op* mice with rh-CSF-1 or expression of a CSF-1 transgene (full length or cell surface), can partially, or fully correct the body weights associated with the *op/op* phenotype. Additionally, serum CSF-1 is elevated in mice in the postnatal period and, together with the finding that injecting newborn mice with rh-CSF-1 causes a significant increase in body weight, it appears that CSF-1 plays an important role in postnatal growth. Therefore, CSF-1 may be a useful therapy in premature human infants. Since the domestic pig has many similarities with humans, especially in terms of innate immune responses, this provides an alternative to traditional rodent models to evaluate the effect of CSF-1 and anti-CSF-1R antibodies on postnatal growth.
Materials and methods: Full length functional porcine CSF-1R (Met1 * Cys969) was expressed in the factor-dependent Ba/F3 cell line and used in conjunction with an Fc-CSF-1R protein to inoculate four adult male BALB/c mice every 28 days for a total of four injections.
Results: Hydridoma cells were generated by fusion of mouse spleen cells with immortal Sp2/0 cells and supernatant screened by indirect ELISA for the production of pig specific anti-CSF-1R antibodies. Positive hybridoma cells were subcloned and assessed by FACS for their ability to bind porcine CSF-1R both in its native state (PBMC and BMC) and on the cloned CSF-1R cell line. Additionally, MTT assays demonstrated the competitive binding properties of the anti-CSF-1R antibodies. The cross-species reactivity of these antibodies has also been investigated.
Conclusions: In conclusion, we have developed porcine anti-CSF-1R antibodies that can be used in a variety of applications and ultimately to assess the role of CSF-1 in the postnatal growth of pigs.

P1513
Versatile generation of human pMHC-specific monoclonal antibodies

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Purpose/Objective: Monoclonal antibodies specific for peptide/major histocompatibility complex (pMHC) specifically expressed by tumors or pathogens-infected cells can be exploited for both diagnostic and immunotherapeutic purposes, in particular for the targeted delivery of toxic agents or immune potentiators. We herein describe a novel versatile approach allowing generation of fully human pMHC-specific monoclonal antibodies.
Materials and methods: We recently set up a pMHC-multimer-based strategy allowing ex vivo detection and flow cytometry sorting ofrare pMHC-specific T cells, present at frequencies as low as 1 in 10^6 from human blood sample (Legoux *et al.*, 2010). Through a similar 8-color flow cytometry approach, we could isolate B cells (CD3-, CD19+) stained by pMHC fluorescent multimers in a peptide dependent fashion. The antibody genes of purified B cells were then amplified by single cell RT-PCR, cloned into expression vectors and transfected into a human cell line. The expressed antibodies were then purified and assayed for binding specificity.
Results: Using this technique, we have been able to generate human antibodies specific for HLA-A2 molecules loaded with particular immunodominant peptides of viral or tumor origins.
Conclusions: This protocol can be performed starting from small blood samples of even non-immunized donors, and can be extended from the generation of human monoclonal antibodies directed against any peptide-MHC specificity to virtually any defined antigen. Our entire strategy used established techniques but is novel in its specificity, sensitivity and applications.

Poster Session: Advances in Biologics 2 — New Developments

P1515

3-dimensional laser structured scaffolds improve macrophage adherence and antigen-specific response

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Purpose/Objective: The aim of the present study was to investigate whether 3D micro and submicron laser textured transplantable biomaterial scaffolds with tunable morphology and chemistry could support macrophage adherence, antigen presentation and specific antibody response development.

Materials and methods: Differences of the morphological features of the candidate biomaterials were advantageously achieved by tuning the laser structuring parameters resulting in scaffolds of gradient micro- and nano-geometries and 3D porosity and hydrophilicity. Macrophages were isolated from BALB/c mice and cultured upon 3D surfaces. Macrophage-coated scaffolds were seeded with antigen (Human Serum Albumin, HSA) and subsequently T_H cells, isolated from BALB/c mice, were introduced to the culture. The absorbance and recognition of macrophages and T_H cells on the 3D surfaces was revealed by SEM and Confocal Microscopy. The system's activation was revealed by Elisa experiments, by measuring the secretion of interleukins IL-2, IL-4 and specific antibody production in cell culture supernatants.

Results: SEM microscopy experiments revealed that less rough 3D hydrophilic scaffolds surfaces were more favorable to macrophage adherence as compared to flat or high roughness scaffolds. Under these conditions cells conserved their morphology and structural properties. Cells were identified as macrophages and T_H, using specific cell markers and by confocal microscopy analysis. T cell activation was demonstrated by detection of IL-2 and IL-4 in culture supernatants, while the development of antigen specific response was proved by detection of HSA-specific antibody production.

Conclusions: The 3D scaffolds displaying the capacity to exquisitely control the size of pores, the micro/nanotopography, the surface chemistry and distribution of bioactive species, represent significant progress beyond the current state of the art enabling a multi-parametric assessment of various factors affecting cell behavior, with far-reaching implications human health.

P1516

A novel carbon monoxide-releasing molecule ALF421 attenuates the development of autoimmune diabetes in mice induced by multiple low doses of streptozotocin

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Purpose/Objective: Carbon monoxide (CO), a by-product of heme catabolism by heme oxygenase, has recently been demonstrated to have potent anti-inflammatory and anti-apoptotic effects. ALF421 is a novel compound designed to liberate controlled quantities of CO in the cellular systems. However, its biological activity in autoimmune type 1 diabetes (T1D) has not been examined so far. Therefore, present study was conducted to investigate possible therapeutic value of ALF421 in the animal model of disease and to explore potential cellular mechanisms involved.

Materials and methods: T1D was induced in C57BL/6 mice by multiple low doses of streptozotocin (5×40 mg/kg). ALF421 (2 and 4 mg/kg) was administered as continuous or 8-day treatment as either prophylactic, early, or late therapeutic regimen. Disease development was evaluated by measuring blood glucose level on a weakly basis. Splenocytes were harvested on day 8 after T1D induction from ALF421-treated or vehicle-treated mice and cytokine levels in 48 h splenocyte-derived culture supernatants were measured by ELISA. Pancreatic islets isolated from healthy C57BL/6 mice, as well as mouse (MIN) and rat (RIN) insulinoma cell lines were exposed to proinflammatory cytokines (IL-1β+IFN-γ+TNF-α, 10 ng/ml each) in the presence or absence of ALF421 (range 12.5—100 μM) and analyzed for their survival by MTT assay.

Results: Administration of ALF421 during diabetes induction, or even after the induction of the disease, suppressed the development of hyperglycemia. *In vivo* treatment with ALF421 down-regulated the *ex vivo* secretion of proinflammatory cytokines (IFN-γ, IL-2, IL-6, IL-1β, and TNF-α), while IL-10, IL-17 and IL-4 production were not affected. *In vitro* studies revealed that ALF421 in a dose-dependent way reduced cytokine-induced cell death in three beta cell types tested (mouse islets of Langerhans, as well as MIN and RIN insulinoma cells), but did not affect basal cell viability.

Conclusions: Although the molecular mechanisms involved in the drug action remain to be established, our results suggest that the observed beneficial effect of ALF421 in the disease process could be attributed at least partly to the interference of ALF421-released CO with cytokine-mediated pro-apoptotic stimuli within endocrine pancreas.

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P1517

A novel platform to screen for carbohydrate-based adjuvants and immunomodulators

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Purpose/Objective: C-type lectin receptors (CLRs) are pattern recognition receptors mainly expressed by antigen presenting cells. CLRs specifically recognize carbohydrate structures present on pathogens, but also self-antigens. The primary interaction of a dendritic cell (DC) and a pathogen shapes the following immune response. Thus, searching for immune modulators that can either enhance or modulate an initiated immune response is of high importance. The objective of this study was to develop a glycan array-based platform to screen for carbohydrate ligands of CLRs and to evaluate their immune modulatory properties *in vitro* and *in vivo*.

Materials and methods: A carbohydrate microarray was prepared consisting of synthetic amine- and thiol-functionalized carbohydrates, including high-mannose structures, phosphatidylinositol mannosides, heparins, lipoarabinomannans, sialylated oligosaccharides, blood group antigens, and tumor antigens. The respective CLR carbohydrate-recognition domains (CRDs) were eukaryotically expressed as fusion proteins with the F_c fragment of human IgG_1 molecules. MGL1-, DCIR-, MCL-, OLR1-, MICL-, CLEC2-, CLEC9A-, and CLEC12b-hF_c were used in this glycan array to screen for carbohydrate ligands. Identified CLR ligands were covalently coupled to the model antigen ovalbumin (OVA). These OVA-carbohydrate conjugates were used in DC/T cell co-cultivation assays to stimulate transgenic T cells *in vitro*.

In addition, mice were immunized with these conjugates to identify immune modulatory CLR ligands *in vivo*.
Results: By using these CLR-Ig fusion proteins in the carbohydrate microarray, several novel glycan structures were identified as binders of CLRs. DC/T cell co-cultivation assays revealed that some of the identified carbohydrate ligands modulated T cell activation and cytokine production. These *in vitro* results were confirmed by immunization studies in C57BL/6 mice indicating that the platform presented here is indeed suitable for the identification of immune modulatory carbohydrate ligands of CLRs.
Conclusions: We here present a platform that brings together CLR ligand identification and their immunologic evaluation. This platform is a useful tool for the development of adjuvants and immune modulators.

P1518
Aedes aegypti saliva as a therapeutic option in the treatment of experimentally induced inflammatory bowel disease

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Purpose/Objective: Inflammatory Bowel Disease (IBD) is a chronic inflammatory disorder characterized by an imbalance between inflammatory and regulatory responses at the gut mucosa. Current treatments are not totally effective and novel therapies are still needed to control IBD. Since mosquito saliva is a source of immunomodulatory molecules, the aim of this study was to evaluate the impact of *Aedes aegypti* salivary gland extract (SGE) in the treatment of experimental IBD.
Materials and methods: To induce colitis C57BL/6 male mice were exposed to 3% (w/v) Dextran Sulfate Sodium (DSS) uninterruptedly in drinking water during nine consecutive days or for two cycles of 9 days with an 11-day interval before the second exposure. From 5th until the 8th day, animals were treated with 1, 3 or 5 μg of *A. aegypti* SGE in 0.1 ml of PBS. Negative control group received only PBS. Mice were daily evaluated for mortality and clinical signs of the disease such as body weight, bloody stools, hiporeactivity and piloerection. The production of cytokines (IFN-γ, TNF-α, IL-4, IL-17 and IL-10) was evaluated by ELISA on gut homogenates of DSS-exposed mice, in the presence or absence of saliva treatment.
Results: The results showed a dose-dependent improvement of the clinical score and recovery of animals treated with EGS. Reduced disease signs were observed in the presence of 3 or 5 μg treatment in the first challenge with DSS, with a lower score in the 5 μg group on days 14 and 15 after initial DSS exposure. During the second DSS-challenge mice treated with 5 μg of SGE showed later and overall reduced clinical signs when compared to the other groups, along with a better recovery at the end of this period. In addition, mortality was lower in 3 and 5 μg groups when compared to the others. Although we did not observe any difference in IL-4, IL-17 or IL-10 in the gut, the improved clinical presentation of the disease could be related to the modulation of cytokines interplay in the intestine, since the 5 μg treated group produced less Th1 or pro-inflammatory cytokines such as IFN-γ and TNF-α in the large bowel when compared to colitis non-treated mice.
Conclusions: These data suggest that *Aedes aegypti* SGE have a beneficial effect when used in the treatment of experimentally induced IBD.

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P1521
C-terminal peptide of prothymosine alpha detected by SELDI-ToF-MS affinity capture technology

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Purpose/Objective: C-terminal peptide of prothymosine alpha [ProTα (100−109)] is a possible early apoptotic marker. We have developed a highly purified polyclonal antibody against the peptide in order to investigate the earliest apoptotic events associated to bacterial infections.
Materials and methods: SELDI-ToF-MS protocols. Chips: NP20 (neat protein) Q10 (weak anion exchanger) and CM10 (weak cation exchanger); matrix CHCA and SPA; several binding buffer pHs; high and *low intensity* laser conditions and several acquisition settings.
Reagents: Antibody Anti-ProTα (100−109); ProTα (100−109); control peptide; Protein A.

Method: Pre-coating of the chips with/without protein A for antibody fixation on the chip; overnight Ab-Ag preincubation; standard chip preparation.
Results: Optimum concentrations of Ab and peptides in the pre-incubation experiments were prior tested in competitive ELISA. Pre-coating with protein A was intended for a better/oriented binding of antibodies. This treatment of chips induced similar spectra with the un-coated conditions. The best spectra were obtained using binding buffer pH = 7. Testing several chip chemistries (see methods), much to our surprise, the NP20 chip did not perform at all in our experiments. In both high/low laser intensity spectra, the antibody, wheatear in Ab-Ag complex or alone, displayed several fragments at m/z 33 666−33 477; 67 419−67−67 148 and 130 500−130 600. In contrast to the reproducible Ab peaks, protein A had a quite random peaks spectrum when comparing triplicate spots or overall duplicate experiments. We have obtained the best results on Q10 chips, ProTα (100−109) being detected as a sharp peak of m/z 1207−1209. Control peptide (scrambled peptide, with identical aminoacid composition but in a different sequence) was detected 7.5 times less that the specific one when the complex Ab-scrambled peptide was subjected to MS.
Conclusions: We have shown that SELDI-ToF-MS can be used as an affinity capture technology to detect Ab-Ag specific complexes.

Optimization of the protocol showed that weak anion exchanger chip gives the best peptide spectra.

ProTα (100−109) displayed a m/z 1207−1209 concordant to the prior published MS data and was detected in the Ab complexes, mass spectrum pulling out the relevant peptide from a specific Ab-Ag complex.

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P1522
Characterisation of antidrug antibodies (ADA) against therapeutic interferon beta

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Purpose/Objective: A significant proportion of multiple sclerosis patients undergoing therapy with interferon beta develop neutralising antibodies against this drug. In this study, our objectives are to cha-

racterise antidrug antibodies to therapeutic interferon beta and to test the potential for differential neutralising capacity of the major drug-specific immunoglobulins.
Materials and methods: Plasma samples from 19 MS patients and 10 age-matched healthy controls were obtained under informed consent from The Walton Centre for Neurology and Neurosurgery, Liverpool, UK. Total and interferon beta specific IgM, IgG and IgG subclasses were quantified by ELISA. Semi-quantitative assessment of neutralising potency of drug-specific antibodies was performed by a bioassay based on the interferon beta responsive Myxovirus-A (MxA) protein. MxA protein levels were measured by immunoblotting.
Results: Interferon beta specific IgG but not IgM was detected and quantified in 8/19 clinical samples. ADA in 7/8 samples neutralised interferon beta. The major interferon beta-specific IgG subclasses were IgG1 and IgG4.
Conclusions: Our data suggests that the interferon beta-specific neutralising antibody profile is dominated by IgG1/IgG4 response in multiple sclerosis patients. Profiling the drug-specific neutralising antibody responses could provide insights into the quality and potential mechanisms that underlie emergence of antidrug antibodies to biologics.

P1523
Development and characterization of an immunosensor for the determination of oxidized low density lipoprotein (ox-LDL)

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Purpose/Objective: The oxidized lipoproteins, especially the oxidized low density lipoprotein (ox-LDL) are present in the plasma of patients with atherosclerosis and have been attributed an important role in illness development. Therefore, the determination of ox-LDL in plasma is essential not only to investigate its relevance for the atherosclerotic diseases, but also to contribute to disease diagnosis.

Thus, this work aims to couple monoclonal antibody to biosensing technology, and produce an immunosensor capable of fast and accurate detection/quantification of ox-LDL in point-of-care.
Materials and methods: oxLDL was prepared and employed to produce anti-oxLDL monoclonal antibodies by hybridomas cells (previously obtained by our group), which were cultured in DMEM with fetal calf serum, glutamine and gentamicin. The monoclonal antibodies were purified by affinity chromatography using Protein G.

The immunosensor was set-up by self-assembling cysteamine (Cys) on the gold (Au) layer (4 mm diameter) of a disposable screen-printed electrode. Antibodies were let react with N-Hydroxysuccinimide and ethyl (dimethylaminopropyl) carbodiimide, and subsequently incubated in the Au/Cys. All these steps were followed by electrochemical techniques.
Results: Three monoclonal antibodies showed the ability of detecting exclusively ox-LDL antigen. All these structures are to be involved in the construction of the immunosensing platform.

Employing Cys self-assembling on gold is a successful way of binding the antibody to a physical support. One of the difficulties around this process is to avoid non-oriented binding of the antibody, which could prevent antigen access to the Fab region. So, the *COOH groups were previously activated, ensuring that mostly the Fb region is involved in connecting the antibody. After optimization of all chemical and physical variables, this sensor will be tested in patients plasma of hypercholesterolemic, with or without atherosclerosis, and normocolestolemics.
Conclusions: The use of monoclonal antibodies on a biosensing platform seems to be a very attractive approach and is expected to provide very important information, correlating the level of ox-LDL fraction with the atherosclerosis disease, in a simple, fast and cheap way. After complete optimization, this device has the potential to improve the current diagnosis of atherosclerosis.

P1524
Evidence for membrane-bound lactoferrin as a pattern recognition receptor on macrophages

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Purpose/Objective: Lactoferrin (LTF) is a soluble glycoprotein of the transferring family well known as a bacterial LPS-chelating agent in biological fluids of mammals. Although it has previously been found on the surface of neutrophils, whether cell surface LTF (scLTF) could function as a receptor for LPS or other pathogen-associated molecular patterns (PAMP) has not been investigated. The present study focuses on the role of csLTF as a pattern recognition receptor (PRR) on macrophages.
Materials and methods: THP-1 cells and peritoneal macrophages, freshly collected from C57BL/6, CD14-knockout or TLR4-knockout mice, were stained with fluorecence-labeled Abs against LTF, MYH9, CD14, or isotype controls, followed by follow cytometric analysis or confocal laser scanning microscopy. For function assays, macrophages were cultured for 48 h in wells precoated with LTF-Abs, or control Abs and then concentration of TNF-α and NO in the culture supernatant quantitated using ELISAs. Routine immunoprecipiation and siRNA inhibition protocols were followed.
Results: Flow cytometric analysis detected LTF on the surface of primary murine macrophages as well as monocytic tumor line cells. Expression of csLTF was greatly reduced in macrophages from CD14-, but not TLR4-, knockout mice. Immunoprecipitation using anti-LTF-Abs pulled down LTF together with non-muscle heavy-chain myosin-9 (MYH9) and also CD14 from the lysate of peritoneal macrophages. SiRNA inhibition of MYH9 or CD14 also led to down-regulation of csLTF expression. Confocal laser scanning microscopy revealed co-localization of LTF, CD14 and MYH-9 on the membrane surface of the cell. Immobilized LTF-Abs were able to induce TNF-α and NO production by wild type, but not $TLR4^{-/-}$ or $CD14^{-/-}$, macrophages *in vitro*. Furthermore, siRNA inhibition of LTF gene expression rendered macrophages refractory to stimulation with LPS or β-glucans.
Conclusions: LTF is anchored to macrophage surface via MYH9 as well as membrane-bound CD14, capable of tranducing activation signals through TLR4. Given that LTF can bind a variety of PAMPs such as LPS, β-glucan, α-mannan and CpG-DNA with high affinity, we propose that csLTF could play an important role as part of a putative broad-spectrum PRR complex on phagocytes.

P1526
Gold nanoparticles downregulate interleukin-1ß-induced biological responses

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Purpose/Objective: Interleukin 1 beta (IL-1β)-dependent inflammatory disorders such as rheumatoid arthritis, psoriasis and many others pose a serious medical burden worldwide, where patients face a life-

time of illness and treatment. Organogold compounds have been used since the 1930s to treat rheumatic and other IL-1β-dependent diseases and, though their mechanisms of action are still unclear, there is evidence that gold interferes with the transmission of inflammatory signalling. The aim of this work was to study the effects of gold nanoparticles on cellular responses to IL-1β.

Materials and methods: THP-1 human myeloid cells were used for *in vitro* studies and C57BL/6 male mice * for *in vivo* experiments. Cellular responses were studied using Western blot analysis, ELISA, scanning electron microscopy in transmission, biochemical assays.

Results: Here, we show for the first time that citrate-stabilized gold nanoparticles specifically downregulate, in a size dependent manner, cellular responses induced by IL-1β both *in vitro* and *in vivo*. Our results indicate that the anti-inflammatory activity of gold nanoparticles is linked to extracellular interaction with IL-1β, thus opening interesting options for further therapeutic applications.

Conclusions: In summary, we have discovered the biochemical mechanisms of AuNP-dependent downregulation of IL-1β-induced inflammatory reactions. This has been addressed on the *in vitro* (cell culture) and *in vivo*. These data allow us to understand the mechanisms underlying the positive therapeutic effects of gold when treating IL-1β-dependent autoimmune disorders (for example rheumatoid arthritis). Furthermore, our study suggests possible clinical implications of AuNPs against different types of widely distributed IL-1β-dependent autoimmune disorders (rheumatoid arthritis, scleroderma, psoriasis etc.).

P1528
Immunological receptors' expression and communication on extravillous trophoblast cell surface

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Purpose/Objective: Molecular and immunological significance of selectively expressed MHC on extravillous trophoblast cells (EVTs) is not well understood. EVTs invade the maternal decidua and are intimately in contact with maternal immune effector cells. The unique MHC class I expression pattern on invasive EVTs shows that classical transplantation antigens HLA-A and HLA-B expression is down-regulated; these cells however, express HLA-E, -F, -G and HLA-C. The expression of MHC class Ib products with limited polymorphism (HLA-G,-E and -F) or class Ia with limited expression (HLA-C) protect EVTs from natural killer (NK)-like large granular lymphocytes (LGL)-mediated attack. In this study, we hypothesise that surface expressed MHC class I molecules interact with each other to modulate the immune-tolerance at the feto-maternal interface.

Materials and methods: Trophoblast model cell lines JEG-3 and ACH-3P were subjected to flow cytometry, immunoblotting and confocal microscopy for expression and quantitation of MHC I antigens . Cell surface HLA-C, -E, -F and -G molecules were detected by L31, MEM-E/07, 3D11 and MEM-G/09 1° antibodies respectively, followed by anti mouse IgG conjugated with either Alexa fluor 488 or 555 2° antibodies. Images were acquired in red and green channels with Nikon confocal microscope using Nyquist resolution with optimised settings (e.g. for Alexa488/555 xy = 55 nm/pixel, z = 140 nm/pixel). This new colocalisation approach is object based, three dimensional and depends on local intensity of highly resolved receptors using sub-pixel resolution. Same algorithm has been used to investigate the level of colocalisation among all MHC I molecules.

Results: Both cell lines expressed cell surface HLA-C, -E and -G but not -F. The object based novel colocalisation analysis revealed that out of total surface HLA-E molecules ~60% of HLA-E molecules are colocalised with HLA-C (~12%) and -G (~47%). Similarly, out of total surface HLA-G molecules ~42% of HLA-G colocalised with HLA-C(~18%) and -E(~24%) and out of total surface HLA-C ~25% of HLA-C colocalised with HLA-G(~14%) and -E(~11%). We also calculated intermolecular distances of colocalised receptors from euclidean distance maps with highest resolution of 15 nm. No significant difference was found on colocalisation in response to progesterone.

Conclusions: Hence, all MHC I receptors may communicate with each other to regulate placentation and facilitate pregnancy progress.

P1529
Monoclonal antibodies from human influenza NP-specific memory B-cells

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Purpose/Objective: Human monoclonal antibodies (mAbs) have previously been derived from individual HIV glycoprotein-specific memory B-cells from infected individuals. The goal of this study was to generate human mAbs from memory B-cells against other viruses.

Materials and methods: Biotinylated recombinant Influenza nucleoprotein (NP) was used to isolate antigen-specific B-cells. ELISpot analysis was performed to determine the purity of the isolated B-cell population. Heavy and light chain variable regions were amplified from single cells and cloned into expression plasmids. MAbs were produced in 293T-cells and analysed in ELISA and Western blot.

Results: 20 mAbs were derived from the blood of three individuals. MAbs recognized linear and conformational epitopes. The paratopes were diverse suggesting that the mAbs are specific for different epitopes.

Conclusions: Purification of protein-specific memory B-cells by immunomagnetic isolation together with antibody gene amplification from single cells can be used to generate recombinant human mAbs from influenza-specific memory B-cells. The technique should be applicable to other viral proteins.

P1530
NK cell — MSC interactions can result in NK cell activation and increased MSC recruitment

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Purpose/Objective: NK cells can have a role in regulating tissue regeneration, particularly through interactions with Mesenchymal Stem/Stromal Cells (MSCs). MSCs can differentiate in different lineages, such as osteoblasts, chondroblasts and adipocytes, secrete bioactive molecules and are immunoregulatory. Since this modulatory capacity does not depend on the donor, clinical strategies using allogeneic cells are being developed for different applications, including treatment of autoimmune diseases and Tissue Regeneration strategies. However, it has become evident that MSCs must be activated to be able to suppress an immune response. And while MSCs can inhibit cytokine-activated NK cells, some reports indicate that MSC can stimulate NK cell cytokine production. Thus, it is crucial to clearly understand the outcome of NK cell and MSC interactions in different contexts. Here, we focused on studying the outcome of bidirectional interactions between freshly isolated NK cells and unstimulated MSCs.

Materials and methods: Human bone marrow MSCs were isolated by adherence and characterized for phenotype and differentiation capacity and peripheral blood human NK cells were obtained by negative isolation (EasySep, StemCell Tech). The outcome of intercellular

interactions was analysed by flow cytometry and imaging flow cytometry. MSC recruitment was determined with Matrigel-coated transwells.

Results: NK cells were found to form conjugates with MSCs, and interestingly MSCs were able to stimulate IFN-γ production and CD107a degranulation by freshly isolated NK cells. Furthermore, MSCs lead to an increased survival of NK cells in culture in the absence of externally added cytokines. On the other hand, it was found that NK cells stimulate MSC recruitment in a chemokine receptor-dependent way, while not impacting on its differentiating capability.

Conclusions: Here, we show that MSCs are capable of activating NK cells, and on the other hand, that NK cells stimulate MSCs recruitment. We believe that taking into account the outcome of NK cell – MSC interactions in different contexts will be crucial in predicting the impact of cell therapies. (Work funded by 'COMPETE – Programa Operacional Factores de Competitividade' (FEDER component) and Foundation for Science and Technology (OE component and QREN-POPH) *PTDC/SAU-BEB/099954/2008 and SFRH/BPD/48533/2008.)

P1531

Non-viral adeno-associated virus-based platform for stable expression of polyclonal antibody mixtures and/or recombinant proteins

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Purpose/Objective: Recombinant polyclonal antibodies represent a promising new therapeutic approach for the treatment of disease. The polyvalent nature of this technology makes it uniquely suited for the control of diseases in which selection for the outgrowth of resistant populations is a growing problem, including antibiotic resistant infectious disease and cancer. However, their complexity also represents a distinct manufacturing challenge. The objective of this work was to develop a rapid, reliable method for the manufacture of polyclonal antibody mixtures of specific and stable composition.

Materials and methods: We have developed a recombinant protein expression platform based on Adeno-Associated Virus (AAV), which demonstrates a high rate of non-random integration and allows the rapid generation of stable antibody-expressing cell populations. HEK 293 suspension cells were cotransfected with an antibody expression plasmid and an AAV virus-derived Rep integrase plasmid, resulting in stable integrants in ~10% of cells. Cells were selected in bulk to generate stable cell populations each expressing a single antibody. Following selection, populations expressing individual antibodies were mixed to generate polyclonal cell cultures for long term expression studies. Cultures were followed over time by Luminex and HPLC for stability of expression and ratios of the component antibodies.

Results: Polyclonal cultures maintained consistent expression levels and stable ratios of component antibodies up to 100 days. Cultures showed remarkable reproducibility following cell banking and antibody ratios were maintained following batch purification. Ratios of component antibodies could be manipulated to achieve desired composition.

Conclusions: Using this method, cell populations expressing antibodies with diverse antigen reactivities can be mixed to produce a stable polyclonal cell population of any desired composition within 30 days. Therefore, this AAV-based expression platform represents a predictable, reproducible, quick and cost effective method to manufacture recombinant polyclonal antibody therapies and other recombinant protein mixtures.

P1532

Outer membrane vesicles of *Neisseria meningitidis* as a new antigen preparation for *Escherichia coli* (STEC)

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Purpose/Objective: *E. coli* (STEC) disease represents an important emerging group of food-borne pathogens. Domestic animals, particularly cattle and sheep, have been identified as natural reservoirs of STEC . Hide contamination occurs from direct or indirect faecal contamination in cattle production and lairage environments. The cause of super shedding by infected cattle, which allows certain individuals to transmit disease at a much higher rate than others, is not known . In Brazil, cases were detected. There are currently no vaccines available that can prevent STEC infection. Production of verocytotoxin or Shiga-like toxin (Stx), particularly Stx2, is the basis of haemolytic uremic syndrome (HUS. The aim of this study was to determine the value of the detoxified Shiga toxins Stx1 and Stx2 (toxoids) from *E. coli* and *N. meningitidis* outer membrane proteins (OMPs) as new antigen preparation.

Materials and methods: The meningococcal group B strain derives from an epidemic in Brazil. OMPs were extracted by treatment of bacteria with 0.5% deoxycholate in 0.1 M Tris-HCl buffer (pH 8.6) containing 10 mM EDTA and purified by differential centrifugation. *E. coli* strains obtained from clinical samples from the state of Baia, Brazil. They are designated as (C7-88) O157:H7 (Stx1) and (1189) ONT:H49 *stx2+stx2vb-hb* (Stx2). All *E. coli* bacterial strains were grown in LB broth. The toxins were detoxified . Briefly, bacteria were centrifuged at $5000 \times g$, and the supernatant was filtered through a 0.45-μm membrane. To evaluate the toxoids and the neutralising activity of anti-Stx1 and Stx2 (OMPs sera), a neutralisation assay using HeLa (CCL-2) cells was performed.

Results: The production of verocytotoxin or Shiga-like toxin (Stx), particularly Stx2, is the basis of haemolytic uremic syndrome (HUS), a frequently lethal outcome for subjects infected with Stx2-producing enterohaemorrhagic *E. coli* (STEC) strains. Our study shows that mice immunised with two doses of the toxoids together with *N. meningitidis* OMPs induced anti-Stx1 or anti-Stx2 antibodies that could neutralise the toxins *in vitro*, at a concentration of 0.75 pg/200 μl/well for Stx2 and 0.39 pg/200 μl/well for Stx1.

Conclusions: *N. meningitidis* may represent a new, nonliving bacterial antigen preparation for use in vaccine as adjuvant in a vaccine against STECtoxin . It has excellent safety properties, high immunological potential and induces production of IgG antibodies capable of neutralising *E. coli* toxins.

P1533

Overcoming multidrug resistance with inhibitor of ABC transporters bound to HPMA copolymer carrier as a novel therapeutic approach in cancer treatment

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Purpose/Objective: Intrinsic or acquired multidrug resistance (MDR) of cancer cells is one of the major obstacles in chemotherapeutic treatment of tumors. Investigated inhibitors of ABC transporters act nonspecifically on the cells of the body, which may be limiting for their clinical use.

The aim of this study was to examine the potential of novel polymeric therapeutics based on *N*-(2-hydroxypropyl) methacrylamide

(HPMA) copolymers bearing either anticancer drug, inhibitor of ABC transporters or both. Binding to the polymeric carrier prolongs blood circulation of the drug and overcomes nonspecific side effects on healthy tissue due to the passive accumulation in tumor tissue (EPR effect). Here we tested the inhibitory activity of three derivatives of reversin 121, which can be bound to HPMA copolymer through a defined biodegradable bond, and we prepared reversin 121 bound to HPMA copolymer carrier.

Materials and methods: The inhibitory effect of unmodified reversin 121 and its derivatives was analyzed by the calcein efflux assay in sensitive mouse P388 cell line and its doxorubicin-resistant counterpart P388/MDR. The cytostatic activity of doxorubicin in both cell lines was tested by [^{3}H]-thymidine incorporation assay.

Results: The results showed that only the derivative of reversin 121 modified with 5-methyl-4-oxoheptanoic acid (MeOHe) increased calcein accumulation to the same extent as unmodified reversin 121 in doxorubicin resistant P388/MDR cells, while neither had any effect in sensitive cell line P388. Similarly, the cytostatic activity of doxorubicin was significantly enhanced by unmodified reversin 121 and reversin modified with MeOHe in resistant P388/MDR cells. Also, an increased cytostatic activity of doxorubicin in P388/MDR cells was seen after incubation with reversin 121 bound to HPMA copolymer. In sensitive line P388, the cytostatic activity of doxorubicin was not affected after incubation with reversin 121, derivatives of reversin 121, nor with reversin 121 bound to HPMA copolymer.

Conclusions: Here we determined that a derivative of reversin121 modified with MeOHe is suitable for binding through a biodegradable bond to HPMA copolymer. Such conjugate exhibits an inhibitory activity to ABC transporters and can be useful in the attempt to overcome the multidrug resistance in cancer.

P1534

Peripheral allergic inflammation affects hypothalamic pituitary adrenal axis function and behavior in a neuropeptide and neurotrophin dependent manner

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Purpose/Objective: An altered reactivity of the hypothalamus pituitary adrenal axis (HPA) has been observed in various patients with chronic humoral inflammatory diseases and in chronically stressed alike. It is associated with an increased production of cytokines such as interleukin (IL) 4, IL 5 or IL 10 and was therefore considered to participate in disease development and aggravation. Allergic inflammation of the skin is one such example. However, the question remained, if the enhanced cytokine production was the cause or the result of enhanced allergic inflammation under stress. Also, additional stress mediators such as substance P (SP) or nerve growth factor (NGF) have evolved and may play a key role in the inflammatory stress response.

Materials and methods: We here employed a combined model of inflammatory and perceived stress in syngeneic C57BL/6 mice to investigate HPA function and interaction with neuropeptidergic and neurotrophin stress mediators.

Results: Atopic dermatitis-like allergic dermatitis (AlD) and control mice showed no significant difference in the total number of activated cFos+ neurons in the hypothalamus. The presence of AlD is therefore not affecting basal HPA function. However, a solitary increase of cFos+ neurons was found in the dorsomedial nucleus at bregma level 1.58 in AlD mice and could be blocked by treatment with SP-neutralizing NK-1 blocker. Exposure to 24 h of noise-stress significantly increased the number of cFos+ neurons in the hypothalamus of control mice as expected. By contrast, AlD reduced the stress-induced increase in the number of cFos+ neurons in the hypothalamus. It also reduced stress-induced peripheral corticosteroid levels alongside with an increased hypothalamic PPT1-mRNA level (endcoding Substance P). As a consequence, stressed AlD mice displayed decreased exploratory behavior in the elevated plus-maze. Increased hypothalamic Substance P mRNA in stressed AlD mice could be reversed by peripheral injection with NGF-neutralizing antibodies.

Conclusions: Taken together, peripheral inflammation derived neurotrophin alters SP expression in the hypothalamus of AlD mice with consequences for HPA function and behavior.

P1535

Phenotypisation of microparticles in heparin-induced thrombocytopenia

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Purpose/Objective: Heparin-induced thrombocytopenia (HIT) with thrombosis is a serious complication of heparin use. Microparticles (MPs) are cellular signalling vesicles released during cell activation or early apoptosis that play an important role in vascular in-flammation, atherosclerosis and thrombosis. MPs expose phosphatidylserine, a procoagulate phospholipid made accessible after membrane remodelling and tissue factor, the initiator of blood coagulation at the endothelial and leukocyte surface. They are present in low concentrations in normal plasma but increased levels are generated by PF4-heparin-antibody interaction. Increased numbers of platelet-derived MPs, which are produced in a heparin-dependent manner, could be a useful biomarker for diagnosis of HIT. The aim of our study was quantification and phenotypisation of MPs in the platelet poor plasma of HIT patients. Additionally we wanted to determine whether MPs could be useful biomarkers for diagnosis of HIT.

Materials and methods: Studies were performed using sera from fourteen patients with HIT who met the clinical criteria. In all patients the diagnosis of HIT was confirmed by using ELISA and functional assays. The results were compared to twenty-one healthy controls. MPs were isolated from plasma by differential centrifugation. Flow cytometry was used to measure the number of MPs, surface exposure of procoagulant phosphatidylserine on MPs (by Annexin V staining), the expression of platelet activation marker * P selectin (CD62P), CD42b and CD42a (glycoprotein Ib and IX) and tissue factor (TF-CD142).

Results: HIT patients displayed higher levels of circulating MP ($P = 0.018$), and higher percept of platelet delivered microparticles (PDMP) which express CD42a+ CD31+ and annexin V ($P = 0.01$), CD42b+annexin V+ ($P = 0.01$), and CD62P+ CD41+ and annexin V+ ($P < 0.01$), and CD142+ (i.e. tissue factor [TF]) and annexin V+ ($P < 0.001$).

Conclusions: Measurement of PDMP in combination with current available assays may be useful for diagnosis of HIT.

P1537

Study of the antigenic cross-reactivity between *Neisseria meningitidis* and commensal *Neisseria* species using (DDA-BF) as adjuvant

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Purpose/Objective: Outer membrane vesicles (OMVs) from *N. lactamica* have emerged as an alternative to vaccination against meningococcal disease .Bilayer fragments of the cationic lipid dioctadecyldimethylammonium bromide (DDA-BF) can be obtained by dispersion of the white powder in an aqueous solution at low ionic strength after sonication at a temperature below 60°C. DDA-BF can be used as an adjuvant, and its main advantage is that DDA-BF requires a lipid concentration lower than the concentrations traditionally used in liposomal formulations .Aluminum compounds remain the only adjuvant that is licensed worldwide.

Materials and methods: Complexes of 25 μg of OMVs of *Neisseria lactamica* in 0.1 mM of DDA-BF were colloidally stable, exhibiting a mean diameter and charge optimal for antigen presentation. Immunogenicity tests for these complexes were performed in mice. In the present study, the immunogenicity of the OMVs of *N. lactamica* was tested in association with fragments of the lipid bilayer of dioctadecyldimethylammonium bromide (DDA-BF) used as adjuvant. In addition, DDA-BF was compared to alum. The evaluation of the cross reactivity of the serum of the animals was performed 45 days after the first immunization. By Dot-ELISA they were tested against different meningococcal strains 39 strains of serogroup A, 42 of serogroup C (1972−1974), 120 of serogroup B (1990−2007), 120 of serogroups B, C, W135, Y (2011−2012) and 50 commensal Neisseria species of Brazil. By Immunoblot we analysed the cross reactivity againstOMVsof *N. meningitidis* of different serogroups, serotypes, subtypes and Neisseria species. The cross reactivity of antibodies was evaluated by immunoblot, ELISA and the avidity index (AI) of IgG antibodies produced.

Results: Results demonstrate byDot ELISA for a total of 321 strainsanalyzed 91% of strains of *N. meningitidis* present reactivitywith polyclonal serum from mice immunized with OMVs of *N. lactamica* and DDA-BF compared with 15% reactivity with aluminum. The serum presented reactivity with the20−130 kDa protein of commensal Neisseria species and interestingly recognized OMVs in *N. meningitidis* of 10−85 kDa. Even after only one immunization, the animals immunized with OMV-DDA-BF produced antibodies with intermediate avidity. For OMV-alum, antibodies with intermediate avidity were only found after two rounds of immunization. However, even in animals immunized two times with OMV alone, only low avidity antibodies were ever generated.

Conclusions: To our knowledge, this is the first study showing an immune response induced by DDA-BF and *Neisseria* genera. Interestingly, DDA-BF was superior to alum as an adjuvant for subcutaneous immunization.

P1538

Therapeutic potential of Saq-NO in blood cancers

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Purpose/Objective: First approved HIV protease inhibitor Saquinavir (Saq) showed remarkable potential to decrease the incidence or promote regression of HIV related cancers such as Kaposi's sarcoma and Non Hodgkin lymphoma. Drug developed by the addition of NO moiety to Saq (Saq-NO) overcomes the quality of original drug, preferentially through neutralization of toxicity. Previously we reported that Saq-NO is efficient against wide array of cell lines *in vitro* as well as rodent models of solid tumors. Here we evaluated its potential against different blood cancer cell lines as well as cells isolated from corresponding pediatric and adult patients.

Materials and methods: Cell viability was estimated by acidic phosphatase test, flow cytometric analysis was done on cells stained by Propidium iodide, Annexin V-FITC/PI, Apostat, DAF-FM or CFSE dye. Mononuclear cells (MNC) from peripheral blood and/or bone marrow were isolated by density gradient centrifugation.

Results: Saq-NO strongly suppressed the viability of Jurkat, Raji, K562 and HL-60 cells *in vitro* with significantly lower IC50 value in comparison to original compound. Sensitivity of blood cancers to Saq-NO overcomes previously determined efficacy of this drug on solid tumors. Reduced cell viability was the consequence of inhibited proliferation while in p53 deficient K562 and HL-60 cells Saq-NO induced caspase dependent apoptosis. Antitumor activity of the drug is not due to the release of NO. Results obtained on cell lines were further evaluated on blood and/or bone marrow MNC of pediatric and adult patients with acute myeloid leukemia and chronic lymphoproliferative disorders. Saq-NO efficiently diminished the viability of MNC and obtained results were in correlation with their malignant potential marked by LDH serum level. Response of MNC from adult patients was stronger in comparison to pediatric patients, while the highest sensitivity to Saq-NO treatment showed MNC from patients with acute myeloid leukemia.

Conclusions: Obtained results showed that Saq-NO possesses extraordinary potential to down-regulate leukemia cell progression indicating the importance of its evaluation in this type of cancers.

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P1539

Use of antibody gene library for the isolation of specific single chain antibodies by ampicillin-antigen conjugates

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Purpose/Objective: Isolation of recombinant antibodies from antibody libraries are commonly performed by different molecular display formats including phage display and ribosome display or different cell-surface display formats. We describe a new plate assay method which allows the selection of *Escherichia coli* cells producing the required single chain antibody by cultivation in presence of ampicillin conjugated to the antigen of interest.

Materials and methods: The method utilises the neutralization of the conjugate by the produced single chain antibody which is secreted to the periplasm. Therefore, a new expression system based on the pET26b vector was designed and a library constructed. The method was successfully established first for the selection of *E. coli* BL21 Star (DE3) cells expressing a model single chain antibody (anti-fluorescein) by a simple plate assay.

Results: Using this plate assay, we could identify a new single-chain antibody binding biotin by growing *E. coli* BL21 Star (DE3) containing the library in presence of a biotin-ampicillin conjugate.

Conclusions: In contrast to methods as molecular or cell surface display our selection system applies the soluble single-chain antibody molecule and thereby avoids undesired effects e.g. by the phage particle or the yeast fusion protein. By selecting directly in an expression strain, production and characterization of the selected single-chain antibody is possible without any further cloning or transformation steps.

Poster Session: Biomarkers & Disease Profiling 1: Learning from Genetics

P1544

A study of a highly conserved intronic region's polymorphism of TIGIT costimulatory receptor in T1D

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Purpose/Objective: TIGIT (T cell immunoglobulin and ITIM domain) is a recently discovered receptor, which share the same ligands with costimulatory receptor CD226 and is expressed on regulatory, memory and activated T cells. In this receptor pair TIGIT pathway leads to T cell inactivation while CD226 transmits activating signal. Genome-wide association study suggested strong association of CD226 locus with type I diabetes (T1D), but there are some evidences, that TIGIT can also play an important role in the development of autoimmune diseases as a negative regulator of immune responses. Currently very little is known about CD226 and TIGIT genetic polymorphisms and their significance in T1D. The aim of the study is to examine genetic polymorphisms of one of these receptors – an inhibitory receptor TIGIT in Estonian population, and look for their association with T1D.
Materials and methods: We compared human and mouse TIGIT gene sequences and found 10 highly conserved intronic regions within the human TIGIT gene. Then we screened these regions for SNP with MAF (minor allele frequency) value over 0.15 and found two SNPs suitable for further investigation. We carried out restriction analysis of one of them: rs62265703 SNP (MAF = 0.1526) located in the second conserved region.
Results: We showed that the SNP MAF is higher in Estonian population (0.254) compared to predicted frequency (0.233) for HapMap CEU population. Statistical analysis revealed no association between the rs62265703 genotype and T1D diagnosis. However in our study population we confirmed that the CD226 polymorphism rs763361 (Gly307Ser) is associated with increased T1D susceptibility.
Conclusions: A further study could assess a functional role of the CD226 polymorphism in the control of T cell activation in both healthy individuals and T1D patients and the TIGIT's impact on an impediment of auto-reactive T cell activation.

P1545

Association of +3179G/A IGF-1R polymorphism and IGF-1 serum level with colorectal cancer development

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Purpose/Objective: The insulin-like growth factor (IGF) system plays a prominent role in cancer development. The IGF-1 receptor (IGF-1R) and its associated signalling pathway is an important growth regulatory pathway that has been implicated in tumour genesis, angiogenesis, metastasis and autoimmune diseases. Through IGF-1R, the proliferative activity of IGF-1 is mainly regulated by the MAPK signalling pathway and its antiapoptotic activity by the PI-3 kinase pathway. It has been demonstrated a significant elevated risk for colorectal cancer (CRC) in relation to increased IGF-1 serum level. Recently, single nucleotide polymorphism located in exon 16 (+3179G/A; rs2229765) of *IGF-1R* gene was found associated with serum levels of IGF-1 and human longevity.
Materials and methods: This study was designed to compare +3179G/A *IGF-1R* genotype distribution in 110 CRC patients to a group of 220 healthy controls. We also investigated serum IGF-1 levels in CRC patients and healthy controls in an association to genotype. IGF-1 serum levels were measured by ELISA and genotyping for the +3179G/A polymorphism was performed by RFLP-PCR assay.
Results: In the study population no significant differences in allele frequencies of investigated polymorphism of IGF-1R between CRC and healthy controls were observed. The higher frequency of heterozygous AG genotype (53% versus 45%; OR = 1.43, 95%CI = 0.82 ÷ 2.50) and lower frequency of GG genotype (29% versus 36%; OR = 0.88, 95%CI = 0.43 ÷ 1.83) was seen in cases versus controls. When CRC patient's group was divided into stages of disease by TNM classification we observed statistically significant increased frequency of AG genotype in III stage compared to controls: 62% versus 45%; OR = 2.81, 95%CI = 1.09 ÷ 7.57; $P = 0.019$. Moreover, AG genotype was overrepresented in advanced (III-IV) CRC compared to early (I-II) stages: 63% versus 43%; OR = 3.37, 95%CI = 1.21 ÷ 9.54; $P = 0.009$, demonstrating that the AG genotype is a risk factor for progression of the disease. According to genotype serum IGF-1 levels decreased depending on number of G allele. Patients with AA and AG genotypes had significantly increased IGF-1 level then patients with GG genotype regardless of disease stage.
Conclusions: Our results showed a relationship between the +3179G>A polymorphism of the IGF-1R and serum IGF-1 with the progression of colorectal carcinoma.

P1547

Deficiency of cyclin D3 a cell cycle gene contributes to development of type 1 diabetes in NOD mice

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Purpose/Objective: To find a new target gene that help to develop a new treatment for type 1 diabetes.
Materials and methods: Mice: KO CcnD3 C57BL6 mice were developed in the laboratory of Peter Siscinski . The mice were then backcrossed 13 times with NOD or NOD/SCID mice to generate aKO micein a NOD background.

Immunofluorescence (IF) and immunohistochemistry (IHC).

Pancreatic sections were incubated with (GP anti mouse INS, IgG anti-Cyclin D3, DAPI) antibodies, and as secondary antibodies anti GP Cy2 and anti IgG Cy5.

Pancreatic islet studies.

Pancreas were digested by collagenase (1.5 mg ml * 1) and isolated in Hanks Buffer salt solution (HBSS). For Cyclin D3 expression, islets per condition were handpickedand digested with trypsin, intensity media of fluorescence (IMF) of Cyclin D3 in CD45-Glut2+ cells were observed by flow cytometry.

Cell culture, transient transfections.

NIT-1 cultures were grown in DMEM supplemented with 25 mM glucose and 10% fetal bovine serum. The cDNA of Cyclin D3 was subcloned under the rat insulin promoter (RIP) in a cassette derived from pBluescript SK (Stratagene, USA) named pBSK-Neo, which confers resistance to Neomycin. The NIT-1 cell line was stably transfected and the resistant clones were selected by Geneticin (G418).
Results: Using the Microarray technology we have identified genes that have downregulation in pancreatic islet endocrine cells during the autoimmune attack progression, one of which encodes for cyclin D3.

The expression of cyclin D3 is greater in a mouse that has no infiltration (NODSCID 11w) compared to a mouse that has (NOD11w). The expression of cyclin D3 is reduced when you transfer at 9 weeks with 10 million total spleen cells to NOD/SCID mouse. The greater the number of total cells of spleen and therefore greater infiltration of pancreatic beta cells decrease expression of cyclin D3. NIT-1 cells transfected with CcnD3 are less susceptible in proinflammatory niche (in medium containing IL-1beta, IFN-gamma or both) to die by apoptosis. Cyclin D3-deficient mice generated faster and with a higher incidence of disease and metabolize glucose more slowly compared to wild type littermates. Human islets express Cyclin D3.
Conclusions: Deficiency of cyclin D3 a cell cycle gene contributes to development of type 1 diabetes in NOD mice. Cyclin D3 is also expressed in human beta pancreatic islets, then, it can maybe help us to develop a new treatment of type 1 diabetes.

P1549
Effect of HLA genotype on the development of neutralizing antibodies to interferon beta in patients with multiple sclerosis

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Purpose/Objective: Development of anti-drug antibodies is a clinical problem associated with the use of different biological therapeutics. A significant proportion of multiple sclerosis (MS) patients treated with interferon beta (IFNβ) develop neutralizing antibodies (NAbs) that can reduce or completely block the therapeutic effect of the drug. Since only a subset of treated patients develops NAbs it is likely that other factors, such as HLA genotype can influence the risk of becoming NAb positive. This study investigates the association of certain human leukocyte antigen (HLA) class I and II alleles with NAb development in a Swedish cohort of IFNβ-treated MS patients and whether or not an association with clinically relevant NAb titers exists.
Materials and methods: Two hundred and thirty NAb-positive and 189 NAb-negative patients with known HLA status were included. NAb titers were measured with RT-PCR (MxA expression). Titers equal or above 150 10-fold reduction units/ml were considered clinically relevant, i.e. reducing the therapeutic efficacy.
Results: HLA-DRB1*15 was associated with clinically relevant titers, especially when correcting for treatment (OR = 2.90, $P = 0.00069$). However, no allele group affected risk of NAbs or clinically relevant titers as much as the treatment formulations. However, The A higher risk was observed in Rebif or Betaferon users compared to Avonex users and HLA genotype was found to influence NAb development differently between users of the different preparations. In Rebif users carriage of *DRB1*15* was associated with development of NAbs and clinically relevant titers. In Betaferon users carriage of *HLA-A*02* was associated with development of NAbs and clinically relevant titers.
Conclusions: The development of NAbs mainly depends on the type of IFNβ preparation used, but is in some part also influenced by HLA class I and II genotype.

P1550
Gene polymorphisms of leptin and leptin receptor are associated with BIM and WHR in obese Mexican woman

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Purpose/Objective: The Leptin, a pro-immflamatory adipokine, is significantly involved in the activation and development of both regional inflammatory responses in adipose tissue and peripheral. The aim of this work, was to determinate the relationship between the polymorphisms G-2548A of *LEP* gen and gln223arg of the *LEPR* gene with the BMI, the body fat distribution, the serum Leptin and the sLR.
Materials and methods: To determinate BMI we used the basic anthropometric parameters, weight and height, for the body fat distribution we used the waist-hip ratio (WHR), as well as the waist circumference of risk for the obese. The polymorphisms were analyzed through its amplification (PCR) and after by restriction enzyme digestion (RFLP s), we used polyacrylamide gels in the electrophoresis to determine the genotypes. The serum concentration of leptin and sLR were measures by ELISA through (R&D) commercial kits.

Statistical analysis: for the description variable we used means and standard deviations, such as frequencies and percentrages. For the inferential analysis we used χ^2 test, *t*-student test, the one way ANOVA (Bonferroni s *post-hoc* test) as well as the Pearson s correlation coefficient.
Results: The obesity group presented high Leptin levels than the normal weight group ($P = 0.018$), as well as lower sLR levels ($P = 0.035$ one tailed). We got significative positive correlation between the BMI and the Leptin concentration ($P = 0.000$, $r = 0.661$) and negative to sRL ($P = 0.018$, $r = -0.331$), also between the sLR and WHR ($P = 0.041$, $r = -0.325$). Respect to the polymorphism, that the individual we found for the *LEP G-2548A* polymorphism, that the individuals with the polymorphic genotype (A/A) presented a higher BMI, into the Obesity group ($P = 0.027$), while for the *LEPR* gln223arg polymorphism, we found in the Normal Weight group that presented the arg/arg genotype had lower sLR concentrations ($P = 0.034$).
Conclusions: The polymorphisms *LEP* G-2548A and *LEPR* gln223arg, are related with the Leptin and sLR levels, as well as with the BMI in the case of *LEP* G-2548A but not with HWE or the cardiovascular risk waist circumference, in women from western of Mexico.

P1551
Genetic risk variants in multiple sclerosis and gene expression in stimulated peripheral blood mononuclear cells

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Purpose/Objective: Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system. Most of the genetic risk loci identified to date are located near genes involved in immune signaling. The objective of the study was to investigate if carriage of MS risk alleles influences the magnitude of expression of immune activation markers upon *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs).
Materials and methods: PBMCs from patients with MS ($n = 34$), clinically isolated syndrome ($n = 7$), other neurological diseases ($n = 27$) and other neurological diseases with inflammation ($n = 26$), were stimulated with LPS and ConA. Relativeexpression of IFN-g, TNF, VAV1, CD74 and TIM1 was measured using Q-PCR with GAPDH as housekeeping gene. The fold change of target gene expression was calculated as the ratio between the expression in stimulated samples and the unstimulated controls. 64 MS-associated SNPs were genotyped using TaqMan OpenArray, while typing of *HLA* genes was done with the Olerup SSP low resolution kit. Differences in fold change of expression stratified by MS risk genotypes were analyzed using the Mann-Whitney test. Benjamini-Hochberg corrections were used to adjust for multiple testing.
Results: The MS risk allele of rs17066096 (*IL22RA2*) was associated with higher fold change of TIM-1 (raw $P = 1.3 \times 10^{-3}$) and IFN-g (raw $P = 4.9 \times 10^{-3}$) expression upon stimulation with LPS. TIM-1 expression was also influenced by presence of rs6062314-TT (*TNFRSF6B*), (raw $P = 2.8 \times 10^{-3}$). The risk allele of rs11129295 (*EOMES*) was

associated with higher fold change of VAV1 (raw $P = 5.5 \times 10^{-3}$) and IFN-g ($P = 9.0 \times 10^{-4}$) expression upon ConA stimulation. None of the associations survived correction for multiple testing at $\alpha < 0.05$. However, there was a trend towards association between rs6062314-TT (*TNFRSF6B*) and TIM-1 (adj $P = 0.091$), rs17066096 (*IL22RA2*) and TIM-1 (adj $P = 0.085$) and rs11129295 (*EOMES*) and IFN-g (adj $P = 0.057$).

Conclusions: We observed a suggestive association between presence of the MS-risk alleles of rs17066096 (*IL22RA2*), rs6062314 (*TNFRSF6B*) and increased fold change of TIM1 expression, as well as increased fold change of IFN-g expression when the risk allele of rs11129295 (*EOMES*) was present. These results warrant replication in a larger, independent material, as well as further investigation of potential functional relationships.

P1552

Immunodetection of human carbonic anhydrase XII as a new potential biomarker of tumor cells

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Purpose/Objective: Carbonic anhydrases (CAs, EC 4.2.1.1) are zinc enzymes that catalyse the reversible hydration of carbon dioxide to bicarbonate. CAs have important physiological and pathological functions in human organism. Some human CAs, such as CA IX are well-recognized tumor markers. Recently, the association of CA XII with human cancers has been demonstrated. The CA XII is a transmembrane protein with an extracellular catalytic domain. It is involved in tumor progression by acidification of the extracellular milieu and regulation of intracellular pH. Recent studies indicate that CA XII is aberrantly overexpressed in breast, cervix, brain cancers, renal carcinomas. Therefore, CA XII might be considered as a useful biomarker of tumor cells and a promising target for specific therapies. The aim of the current study was to develop new monoclonal antibodies (MAbs) against human recombinant CA XII and evaluate their diagnostic potential.

Materials and methods: The extracellular domain of human CA XII was expressed in *E. coli* and used as an immunogen. The MAbs were generated by hybridoma technology. MAb specificity was analysed by ELISA, IHC and flow cytometry.

Results: Seven stable hybridoma cell lines producing IgG antibodies against human CA XII were generated. The MAbs were highly specific to CA XII and did not cross-react with human recombinant CA I, II, III, VII and XIII. In order to demonstrate the diagnostic value of the MAbs, they were employed for the IHC staining of CA XII in human tumor tissue specimens. The MAbs demonstrated a strong and specific reaction with colon and renal carcinoma specimens and did not show any unspecific background staining of the respective normal tissues. Flow cytometry analysis revealed a specific immunostaining of CA XII in human tumor cell lines.

Conclusions: The newly developed MAbs represent a promising diagnostic tool for the immunodetection of CA XII-expressing tumor cells.

P1554

Interleukin-2 gene polymorphism in Turkish patients with Behçet's disease and its association with ocular involvement

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Purpose/Objective: Behcet's disease (BD) is a chronic immune-mediated systemic disease, characterised by oral and genital lesions and ocular inflammation. Several cytokine genes may play crucial roles in host susceptibility to BD, because the cytokine production capacity varies among individuals and depends on the cytokine gene polymorphisms. The association of the interleukin (IL)-2 gene polymorphisms with the susceptibility to BD was investigated in this study.

Materials and methods: DNA samples were obtained from a Turkish population of 97 patients with BD and 76 healthy control subjects. Polymorphisms of IL-2 gene at position -330 and +166 were determined by using the polymerase chain reaction with sequence-specific primers.

Results: In the patients with BD, there was a significantly increased frequency of IL-2 -330 TG genotype. Meanwhile, a significant decrease in the frequency of IL-2 -330 TT genotype was detected in the patient group in comparison to healthy controls. Interestingly, the frequencies of IL-2 -330 TG and IL-2+166 GG was increased in BD patients with ocular involvement, whilst IL-2 -330 TT genotype was significantly decreased.

Conclusions: These results reveal that IL-2 -330 TG genotype may be a susceptibility factor for BD, whereasIL-2 -330 TT genotype seems to display a protective association with BD. Additionally, IL-2 gene polymorphisms might be associated with ocular involvement in BD.

P1556

Isolation of CD4+ T lymphocytes for the unbiased analysis of their gene expression profiles

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Purpose/Objective: Biomarkers are important tools for diagnosis of immune-mediated diseases, their prognosis and the prediction of response to therapy. Cells of the peripheral blood are expected to reflect systemic and local pathogenesis, in terms of their gene expression profile. Microarray analysis of cell-type specific transcriptomes, however, requires isolation of defined cells to high purity, without affecting their gene expression. Here, we analyze the influence of cell sorting procedures on the gene expression profile of human peripheral CD4+ T lymphocytes.

Materials and methods: CD3+CD4+CD45RO+ T cells were isolated from the peripheral blood of healthy volunteers, comparing seven different procedures, including erythrocyte lysis at 4°C and 20°C, ficoll gradient centrifugation, direct and indirect enrichment by magnetic bead assay as well as the use of the transcriptional blocker ActinomycinD. For control, T cells were intentionally activated with phorbol myristate acetate (PMA) and ionomycin. Total RNA was extracted from the cells, and microarray analysis of the transcriptomes was performed using Affymetrix Human Genome U133 Plus arrays.

Results: The transcriptom analysis revealed but minor differences between the isolation procedures. Nevertheless, erythrocyte lysis at

20°C induced more changes of the transcriptome than ficoll gradient centrifugation with an equally long handling step at 20°C and direct enrichment by magnetic bead assay performed best in regard to time and yield. Independent of the isolation procedure, the gene expression pattern of CD4+CD45RO+ T helper cells was however severely impacted by duration and conditions of storage of the samples between venipuncture and cell isolation.
Conclusions: When cells were isolated from fresh blood, their gene expression pattern could be maintained through the isolation procedures tested, as long as the isolation was performed fast and under predominantly cold conditions.

P1557

Restricted immunoglobulin repertoire and evolutionary mechanism of PDGFR-autoreactive memory B cells in systemic sclerosis

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Purpose/Objective: Systemic sclerosis or scleroderma (SSc) is a clinically heterogeneous disease of the connective tissue characterized by vascular, immune/inflammatory and fibrotic manifestations. We have provided evidence that the serum of SSc patients contains stimulatory auto-antibodies (auto-abs) directed to the PDGF receptor (PDGFR) that elicit reactive oxygen species (ROS) and collagen production in human fibroblasts (Baroni SS, NEJM 2006). This study was aimed at analyzing the native immune repertoire of two distinct SSc patients.
Materials and methods: Memory B cells of two SSc patients were immortalized and screened for PDGFR-autoreactive clones. RNA was extracted from each PDGFR-autoreactive B cell line, reverse-transcribed, and amplified with a set of primers designed to analyze the human immunoglobulin (Ig) gene repertoire. Ig variable (V) regions identified in these clones were expressed as human IgG monoclonal abs and characterized through binding and functional assays.
Results: PCR analysis of rearranged Ig genes suggested the presence of a large panel of V heavy (H) and light (L) chain subgroups. However, subsequent sequencing showed a small panel of VH and VL chains in each B cell line: the same variable Ig sequences were recovered repeatedly from independent PCR amplifications of different VH and VL subgroups, and displayed a high frequency of somatic mutations in the complementarity determining regions (CDRs). Ab engineering revealed that four different VL chains identified in one SSc patient conferred diverse biological properties to the unique VH chain identified in the same patient: loss of high affinity binding; PDGFR binding without signaling; PDGFR binding with ROS induction; PDGFR binding with ROS and collagen gene induction.
Conclusions: The repertoire of PDGFR-autoreactive SSc B cells is restricted and somatically mutated. This suggests the hypothesis of a polarization of the immune response towards the autologous PDGFR under the influence of a strong antigenic drive, the nature of which awaits identification. Stimulatory and non-stimulatory anti-PDGFR auto-abs coexist in the same SSc patient, they share a common VH chain and have, therefore, a common origin. The CDRs of the shared VH framework might represent a common trait of SSc patients Ig, and be used as novel biomarkers of this disease.

P1559

The effect of insulin-like growth factor 1 on IL-12p40 and IL-10 production depending on polymorphisms associated with the induced cytokine production

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Purpose/Objective: Relatively recently insulin-like growth factor 1 (IGF-1) and IGF-1 receptor (IGF-1R) is considered to have a role in the regulation of immune function. It may represent an important switch governing the quality and amplitude of immune responses. IGF-1/IGF-1R signalling may also participate in the pathogenesis of autoimmune diseases, although its relationship with these processes seems complex and relatively unexplored. Interleukin (IL)-12 and IL-10 are immunoregulatory cytokines with an antagonistic effect of the T-helper (Th)1/Th2 cytokine balance and provide a functional link between innate and adaptive immune responses. The aim of the present study was to investigate the effect of IGF-1 on induced cytokine production of IL-12p40 and IL-10 by stimulated peripheral blood mononuclear cells (PBMC) isolated from healthy donors. We also investigated the association of cytokine production and single nucleotide polymorphisms (SNPs) that have been reported to show associations with the production of IL-12p40 and IL-10.
Materials and methods: Peripheral blood mononuclear cells (PBMC) from 11 healthy donors were isolated by Histopaque-1077 density gradient centrifugation. After 20 h of resting the cells were stimulated with 100 ng/ml recombinant human IGF-1 with or absent of LPS (1 μg/ml). Supernatants separated after the 6 and 24 h from the stimulation were quantity determinate by ELISA method for IL-12p40 and IL-10. Genotype and allele frequencies were defined by PCR-AFLP.
Results: Our results showed a light down regulation by IGF-1 of induced IL-12p40 production in the presence of LPS (LPS − 360 ± 292 versus LPS/IGF-1 − 313 ± 256 pg/ml). Preliminary data from qRT-PCR showed the same results for the mRNA levels. Donors with *1.2/2.2* genotype for the *IL-12p40pro* polymorphism located in the promoter of the gene have four times high production of IL-12p40 ($P < 0.05$). We observed absence in the induced cytokine production for IL-10 according to IGF-1.
Conclusions: Based on the results we can conclude that IGF-1/IGF-1R signalling pathway is not directly involved in the induced cytokine production of IL-12p40 and IL-10. These results will be supplemented by additional studies on mRNA expression of the target cytokines in PBMCs cultures.

P1560

The role of the TNF -308G/A polymorphism in disease development of pulmonary sarcoidosis in Czech patients

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Purpose/Objective: Pulmonary sarcoidosis is an inflammatory immune-mediated disorder characterized by the presence of granulomas in the lung. Previous studies reported association of tumour necrosis factor (TNF) -308*A allele (rs1800629) with L*fgren's syndrome (an acute form of sarcoidosis, LS) and favourable prognosis in the Dutch population. We, therefore, aimed to determine the distribution of TNF -308G/A polymorphism in Czech sarcoidosis patients and its clinical phenotypes.

Materials and methods: TNF -308 G/A polymorphism was genotyped using MassArray technology (PCR with MALDI-TOF MS detection, Sequenom) in 122 Czech patients with pulmonary sarcoidosis. Patients were further subdivided according to the presence/absence of LS, chest X-ray stage (CXR) and disease development after 2 years (remission/progression). High-sensitivity ELISA was applied to detect TNF-alpha serum levels in order to assess the functional impact of rs1800629.
Results: The TNF -308*A allele frequency in sarcoidosis patients (22%) were similar to those published in healthy Czech population (21%). No association between TNF -308 polymorphism and sarcoidosis as a whole or with subgroups according the CXR-stage was found ($P \geq 0.05$). The TNF -308*A allele was more frequent in patients presenting LS (33%) compared to those without LS (19%, $P = 0.03$). No significant difference in allele frequency was observed between patients subdivided according to the disease development ($P \geq 0.05$). The study of association between rs1800629 and serum levels of TNF-alpha is under evaluation.
Conclusions: We confirmed overrepresentation of TNF -308*A allele in Czech sarcoidosis patients with L*fgren's syndrome. However, we didn't confirm association of the TNF -308*A allele with good prognosis in our patients with sarcoidosis. Further studies on the usefulness of TNF -308G/A genotyping for the prediction of the prognosis in an extended patient cohort are ongoing.

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P1561

The search for candidate genes that characterise susceptibility or resistance to pathogenic mycobacteria

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Purpose/Objective: Resistance and susceptibility to infectious diseases is well-documented in a variety of host species and while many factors contribute to resistance/susceptibility dynamics, a major component is the genotype of the host. *Mycobacterium avium* subspecies *paratuberculosis* can cause a chronic granulomatous enteritis, Johne's disease, in ruminant animals such as sheep, cattle and deer. Over the years the Disease Research Laboratory has observed a pattern of resistance and susceptibility to Johne's disease in some breeds of red deer (*Cervus elaphus*) but the immunological basis of this phenomenon is unknown. The macrophage is an immune cell which is central to all mycobacterial infections and therefore the gene expression response of this cell has been assayed in red deer from a resistant or susceptible genetic background.
Materials and methods: Macrophage cultures were derived from the blood monocytes of 10 resistant and 10 susceptible animals, infected with MAP *in vitro* and the expression of candidate genes assessed by qPCR. Analysis of the transcriptome of macrophages from two resistant and two susceptible animals by the Illumina HiSeq 2000 sequencing system was also undertaken.
Results: The resulting profiles indicate that susceptible animals have a much higher upregulation of inflammatory genes and pathways (such as iNOS, IL-1α, TNFα and IL-23p19) than the resistant animals. This may in fact be over-expression in the susceptible animals leading to an exacerbation of disease symptoms and inefficient clearing of the mycobacterial organism compared to the more controlled upregulation of inflammatory pathways in macrophages from resistant animals.
Conclusions: Work of this kind has never been performed before and has produced data to advance the understanding, both of the biology of JohneÕs disease and of mycobacterial disease in general.

Poster Session: Biomarkers & Disease Profiling 2: Immune-Monitoring

P1563

Adaptive and innate immunity in patients with intracranial infectious and inflammatory diseases

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Purpose/Objective: To study features of the immune responsiveness of patients with different clinical forms of intracranial infectious and inflammatory processes (ICIIP), to assess the clinical relevance of the data obtained and to create a model of the ICIIP immunopathogenesis and some of the typical forms of ICIIP associated with clinical and immune-mediated postinfectious syndrome (CIPIS) in the pathogenesis of ICIIP.
Materials and methods: 88 serum samples of patients with ICIIP confirmed by imaging tests (CT, MRI) or during the surgery (brain abscesses – 55; dural empyema – 25; meningoencephalitis – 8) and 29 clinically healthy donors have been studied. Cytometry was used for immunophenotyping of blood cell subsets; screening of cytokines, for anti-tissue, anti-organ and antimicrobial antibodies (Abs) as well as for B7-H1 biomarker by ELISA and cytometry; identification of infectious pathogens was done by culture and PCR.
Results: Two major forms of ICIIP-associated CIPIS have been identified and confirmed, namely, (1) postinfectious secondary immunodeficiency syndrome (PISIS) – 92% of the total number of patients (including aggressive subtypes: 38% and moderate ones: 54%); (2) PISIS associated with autoimmune syndrome (PISIDAS) * around 10% of patients. Specific features of either of two syndromes have been established, namely, for: (I) *innate* immunity and (II) *effector* link of *adaptive* immunity and *regulatory* mechanisms of the immunity as a whole.
Conclusions: Information obtained from the studies has given a way to define two new classes of criteria to be implemented into clinical and preclinical practice. The first class would include microbiome-based criteria to monitor spectrum and the localization of the microbial gene pool and serologic profile anti-microbial Abs. The second one would include immune-based criteria to predict risks of transformation (*predictive* factor) of acute forms of ICIIP into the chronic ones and development of complications.

P1564

Adiponectin, leptin, resistin – players in driving the rheumatoid arthritis activity

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Purpose/Objective: The adipose tissue by its hormones * adipokines (adiponectin, leptin and resistin) proved to be not only a fat depot but an active player in driving the inflammatory and the immune process. The main aim of the study was to evaluate the impact of the adipose tissue in the assessment of the RA activity.
Materials and methods: A longitudinal, observational, prospective study was performed in the University Clinic of Targu Mures, Romania. 64 patients (pts.) diagnosed with rheumatoid arthritis with medium-high activity (DAS > 3.2) and no cardiovascular or metabolic conditions recorded were included in the study. They were divided into three groups according with their body mass index (BMI). The classical inflammatory biomarkers * erythrocyte sedimentation rate (ESR), C-reactive protein (CRP); the synovial activity measured by a quantitative Color Doppler ultrasound score * Color Fraction (CF); anthropometric measurements such as: body mass index (BMI), the Hoffa fat pad and the adipokines were assessed. The Graph Pad Prism5 statistical software was used to assess the data.
Results: Overall, a positive correlation between the levels of leptin and the BMI was observed ($P = 0.015$, $r = 0.313$) and a negative correlation between Hoffa fat pad and leptin ($P = 0.007$, $r = -0.050$). In the overweight (OW) patient group the synovial activity expressed by the CF was correlated with the levels of adiponectin ($P = 0.006$, $r = 0.639$). The levels of both leptin and adiponectin correlated positively with the Hoffa fat pad in the OW group ($P = 0.001$, $r = 0.723$; $P = 0.041$, $r = 0.500$). The levels of resistin were positive correlated with the CRP ($P = 0.017$, $r = 0.626$). In the obese group a positive correlation was observed between the leptin's levels and the CF ($P = 0.044$, $r = 0.466$) and a negative one with the Hoffa fat pad ($P = 0.020$, $r = -0.574$). In the normal weight patients group a positive correlation was observed between resistin and CF ($P = 0.011$, $r = 0.521$), the ESR and adiponectin ($P = 0.034$, $r = 0.465$), a positive one between Hoffa fat pad and leptin ($P = 0.021$, $r = 0.479$) and negative with adiponectin ($P = 0.006$, $r = -0.551$).
Conclusions: Leptin exerted a pro-inflammatory role in the obese RA patients. Adiponectin and resistin, adipokines rated in previous studies as anti-inflammatory showed an inflammatory behavior in the OW RA patients.

P1565

Aminoglutethimide-induced immune changes: implications for idiosyncratic drug reactions

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Purpose/Objective: As with most aromatic amine drugs, the aromatase inhibitor aminoglutethimide (AMG) is associated with a variety of idiosyncratic drug reactions (IDRs) ranging from skin rashes to serious haematological toxicities. Currently, the mechanisms of IDRs are poorly understood and their rare and unpredictable natures are major issues for drug development that may lead to serious consequences upon market release. AMG-induced IDRs are typically delayed in onset and evidence suggests they are immune-mediated; therefore, the objective was to characterize the changes to the immune system induced by AMG in rodents to gain better insight on the mechanisms of IDRs.
Materials and methods: Male Brown Norway rats were dosed with 125 mg AMG/kg/day by oral gavage for 14 days and various immune changes were monitored in the blood, bone marrow, and spleen.
Results: Neutrophilia was observed as early as 24 h in AMG-treated rats, and this was sustained over the course of treatment despite no changes to the total WBC count because of lymphopenia. This corresponded to increased release of newly formed neutrophils from the bone marrow as determined with BrdU labeling and a significant increase in the M:E ratio. In addition, serum cytokines such as TNF-α, Cxcl-1, and G-CSF were up-regulated in the AMG-treated. In the spleen, increased $CD3^+CD4^+Ki\text{-}67^+$ staining was observed in the white pulp at day 14 of AMG treatment, which suggests lymphocyte proliferation.
Conclusions: These AMG-induced changes are consistent with activation of both the innate and adaptive immune response, and may be typical of other drugs that can induce IDRs. Furthermore, investigation of the balance between immune activation and immune tolerance may be crucial to understanding why most people do not have IDRs when

treated with a drug. This research was funded by grants from the Canadian Institutes for Health Research.

P1566

An analysis of regulatory B and T cells in the peripheral blood of patients with rheumatoid arthritis – using bioconductor packages for flow cytometric analyses

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Purpose/Objective: Bioconductor offers packages for R supporting the complete work-flow to analyse large-scale flow cytometry data. We evaluated the usability of this software and compare the variability between manual and automated analysis using a large data set obtained from the analysis of regulatory B- and T-cells from patients with rheumatoid arthritis (RA).

Materials and methods: PBMCs from 61 patients suffering from RA were isolated and stained for regulatory T and B cells using two panels containing eight markers each. Additionally, isotype controls were included for two non-lineage markers. Data were acquired using a FACS Aria II flow cytometer.

Manual analysis was performed by two independent evaluators using the FACS Diva software (v6.1.2). For automated analysis, R (v2.15.0) and Bioconductor packages (v2.10) were used. Correlation analyses of the percentages of ten cell populations present in the stained and the non-lineage isotype controls were carried out to determine gating robustness.

Results: Manual analysis took 25 working hours before a table was completed that contained ten variables for subsequent statistical analyses. In contrast, the automated data analysis took 15 h only. Manual analysis resulted in median correlation coefficients for the percentages of the various cell populations in the stained and non-lineage isotype control samples of 0.88 and 0.91 for an unexperienced and an experienced evaluator, respectively. Likewise, the automated analysis resulted in a median correlation coefficient of 0.84 without any significant differences between the three analysis groups. So far, we did not find any correlation between the percentages of $CD4^+foxp3^+CD25^+CD127^{+/-}helios^{+/-}$ cells and the percentages of naïve, memory, transitional and regulatory B cells and their expression of CD80 and CD86.

Conclusions: The freely available statistical software R in combination with Bioconductor's flow cytometry packages is well suited to perform high quality flow cytometry analyses and is comparable with manual analysis in terms of gating precision yet is less time consuming for large-scale studies.

P1567

Analysis of serum S100A8/A9 in patients with anti-neutrophil cytoplasm autoantibodies -associated vasculitis

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Purpose/Objective: S100A8/A9, also called Calprotectin is a calcium and zincum binding protein, belonging to S100 protein family, that is mainly released during neutrophil or monocyte activation. It's an important proinflammatory mediator firstly reported to be determinant biomarker to differentiate Inflammatory bowel disease (IBD) from non inflammatory intestinal symptoms. To date, there have been few reports focusing on the clinical importance of S100A8/A9 in anti-neutrophil cytoplasm autoantibodies (ANCA) associated vasculitis. The aim of this study is to determine levels of this protein in sera of patients with this disease and then to analyze their relationship with disease activity.

Materials and methods: The serum concentration of S100A8/A9 was measured, using an enzyme immunoassay, in 27 patients with ANCA-associated vasculitis and in 26 healthy control subjects. Our patients were selected such a way that 16 of them were in full disease flare with high levels of ANCA, while the serology was negative in the remaining 11 patients in remission.

Results: Serum S100A8/A9 wasnot significantly higher in ANCA-associated vasculitis patients than in control group (246 mg/l versus 251, $P = 0.183$). The breakdown of our population into two groups-according to disease activity and ANCA levels, 16 in disease flare and11 patients in remission showed thatincreased levels of Serum S100A8/A9 was notassociated with severity of ANCA-associated vasculitis ($P > 0.05$).

Conclusions: Unlike what has been recently reported that increases in serum levels of S100A8/A9 correlated well with some clinical forms of a few inflammatory diseases such as rheumatoid arthritis, our findings demonstrated the lack of correlation between this serum biomarker and the activity of ANCA-associated vasculitis, pathology in which polynuclear neutrophils are known to be involved in mechanism injury.

P1569

Automated imaging system for the analysis of antinuclear antibodies by indirect immunofluorescence

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Purpose/Objective: Antinuclear antibodies (ANA) are essential biomarkers in the diagnosis of several autoimmune diseases. Although immunoassays in solid phase as ELISA are economic and easier to standardize, they are not able to describe ANA patterns and have less sensitivity than indirect immunofluorescence (IIF) in human epithelial cells HEP2, for this reason the American School of Rheumatology recommends IIF in HEP2 as the technique of reference for the determination of ANA. Following this tendency, technologies for the reading of ANA by IIF are being developed in order to avoid the main disadvantage of the IIF; the variability of results based on the experience of the analyst.

G-Sight Zenit is an automated system of Menarini Diagnostics for the capture of images and the interpretation of IIF tests that allows a semiquantitative analysis and the recognition of ANA patterns in HEP2. The use of these systems could help reduce the great variability in the determination of these autoantibodies.

Objective: Analyze the degree of agreement between the results obtained with the automated system (Zenith G-Sight, Menarini Diagnostics) and the microscope observation for the analysis of the ANA by IIF.

Materials and methods: We analyzed 107 serums from patients being followed by specialized consults. The samples were analyzed in parallel with the automated system Zenith G-Sight from Menarini Diagnostics (Florence, Italy) and with a fluorescence microscope Olympus BX41 (Tokyo, Japan). We used as substrate, slides of HEP2 cells, Immuno-Concepts. (Sacramento, USA) We determined the degree of agreement for pattern and titer of ANA between the images obtained by the G-Sight and the microscopic analysis, by the calculation of Kappa indices using the statistical program SPSSv19.

Results: In the study of the patterns we obtained a kappa index of 0.78, indicating a good degree of agreement between both techniques. In the study of the titer we obtained a kappa index of 0.53, thus the agreement between both techniques was moderate.

Of the 48 samples interpreted as negative/doubtful by the G-Sight, 32 were reported as positive and 16 as negative aftermicroscope observation.

Conclusions: Both methods show a good degree of agreement as far as pattern and titer. Nevertheless, it would be necessary to adjust the rank of negatives/doubtful given by the G-Sight to use this equipment in ANA screening.

P1572

Comparative study between ELISA and chemiluminescence methods for the analysis of specific ENA

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Purpose/Objective: The autoimmunity laboratory plays an essential role in the diagnosis, classification, prognosis and follow up of autoimmune diseases through the detection of serum autoantibodies.

Autoantibodies to extractable nuclear antigens (ENA) anti-Ro/SSA, anti-La/SSB, anti-Sm, anti-RNP/U1RNP, anti-Scl-70/topoisomerase I and anti-Jo-1/histidyl-tRNA synthetase are clinically important in patients with autoimmune diseases such as Sjögren's syndrome, systemic lupus erythematosus (SLE), scleroderma, dermatomyositis and polymyositis among others.

Their analysis is made preferably by enzyme linked immunosorbent assay (ELISA). This method has very good sensitivity and can detect small concentrations of antibodies. Nevertheless, in the last years the development of new chemiluminescent immunoassays is changing the methodology of the autoimmunity laboratories.

Objective: Analyze the degree of agreement between chemiluminescence Zenith-RA method from Menarini Diagnostics (Florence, Italy) and the habitual ELISA from Inova Diagnostics (San Diego, USA) for the analysis of specific anti-ENA, anti-Ro/SSA, anti-La/SSB, anti-Sm, anti-RNP/U1RNP, anti-Scl-70 and anti-Jo-1.

Materials and methods: We analized 85 samples from rheumatology patients using both instruments Zenit-SP+ (ELISA, Inova Diagnostics) and Zenit-RA (chemiluminescence immunoassay, Menarini Diagnostics). Once we obtained the results of the specific anti-ENA we classified our patients in positive or negative according to the cut-off values recommended by the manufacturer and we determine the degree of agreement using the statistical program SPSSv19.

Results: The Kappa indices show a good degree of agreement foranti-Sm, anti-Scl70 and anti-Ro/SSA and moderate degree of agreement for anti-La/SSB, anti-RNP/U1RNP and anti- Jo-1.

Conclusions: Both methods show good degree of agreement in the analysis of specific anti-ENA. Given the advantages of CLIA techniques in front of ELISA (master curve for each lot of calibrators and controls, linearity and continuous access of samples) it could be a valid option for the analysis of specific anti-ENA in the clinical laboratory.

P1574

Development of a conformation-dependent immunoassay for the diagnosis of systemic sclerosis

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Purpose/Objective: The presence of conformational, stimulatory auto-antibodies (abs) against the PDGF receptor (PDGFR) in patients affected by systemic sclerosis (SSc) has been questioned. IgG purification procedures and technical problems in cell-based biological assays and in solid phase binding assays can disrupt agonistic conformational abs and generate artifacts. To solve these issues and to validate the presence of anti-PDGFR abs in SSc patients, we aimed at developing a conformation-dependent immunoassay to detect agonistic anti-PDGFR abs in serum samples.

Materials and methods: (1) Human monoclonal anti-PDGFR autoabs were cloned from memory B cells of SSc patients; (2) different recombinant PDGFR conformers were tested to set up a capture ELISA able to detect serum anti-PDGFR auto-abs; (3) competitive binding assays using anti-PDGFR monoclonal auto-abs and recombinant human PDGF were performed to define the PDGFR epitopes bound by these different ligands; (4) a conformational peptide library spanning PDGFR extracellular domains was generated to validate epitope mapping data; (5) peptides bound by monoclonal auto-abs were synthesized and pre-incubated with agonistic auto-abs to inhibit antibody biological activity.

Results: (1) Monoclonal anti-PDGFR auto-abs generated from SSc B cell repertoire exhibited different PDGFR binding and agonistic properties; (2) a map of extracellular PDGFR functional domains was obtained: PDGFR epitopes bound by stimulatory auto-abs differed from those of non-biologically active auto-abs; (3) pre-incubation of agonistic auto-abs with peptides corresponding to their epitopes inhibited collagen stimulation in fibroblasts; (4) a competitive ELISA based on the inhibitory peptides was established to detect conformational PDGFR auto-abs in serum: this assay allowed discrimination between SSc and control sera with remarkable specificity and sensitivity.

Conclusions: The identification of the epitopes of the stimulatory, SSc-specific, anti-PDGFR auto-antibodies was crucial to develop a conformation-dependent immunoassay discriminating between SSc and control sera. The solid phase binding assay based on these epitopes might be used as a novel tool for diagnosis of SSc and classification of the clinical subsets of disease and related conditions such as Raynaud's Phenomenon.

P1577

Diagnostic value of serum high mobility group box protein 1 (HMGB1) and soluble CD163 (sCD163) levels in brucellosis

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Purpose/Objective: Both $CD4^+$ and $CD8^+$ T lymphocytes play critical roles in immunity to *Brucella*, in part because they secrete IFN-γ and activate the bactericidal functions in macrophages. Therefore, use of markers which can evaluate macrophage activation may have diagnostic and prognostic importance.

High mobility group-box 1 protein (HMGB1) is a late-onset proinflammatory cytokine secreted from activated macrophages. Soluble hemoglobin scavenger receptor (sCD163) is a specific marker of anti-inflammatory macrophages.

The aim of this study was to test potential associations of serum levels of HMGB1 and sCD163 with Brucellosis and its acute, subacute and chronic forms.

Materials and methods: Serum HMGB1 and sCD163 levels in 49 Brucellosis patients (23 acute, 15 subacute, 11 chronic) were compared with 52 healthy control subjects. Brucellosis was diagnosed by a positive blood culture and/or increased *Brucella* antibodies in serological tests in addition to compatible clinical symptoms. Con-

centrations of serum HMFB1 and sCD163 were determined by ELISA according to the protocol of manufacturer.
Results: Both serum HMGB1 and sCD163 levels were significantly higher in Brucellosis patients compared with healthy controls. The median serum HMGB1 level was 77.09 (0.00−182.84) ng/ml in healthy controls, whereas it was 170.65 (13.19−188.23) ng/ml in Brucellosis patients ($P < 0.001$). The median serum sCD163 level was 0.57 (0.21−1.52) mg/l in healthy controls, whereas it was 1.27 (0.37−2.13) mg/l in brucellosis patients ($P < 0.001$). There was no statistically significant difference in serum levels of HMGB1 and sCD163 among acute, subacute and chronic cases with Brucellosis. Additionally, serum HMGB1 levels were positively correlated with sCD163 levels, whilst neither HMGB1 nor sCD163 levels were correlated with CRP, WBC and sedimentation values.
Conclusions: Our study demonstrated that the levels of HMGB1 and sCD163 may be diagnostic markers for Brucellosis, but none of them can be used for differentiating three different forms of disease (acute, subacute, chronic). On the other hand, studies with larger series of patient populations are necessary to confirm the biological significance of our results. Also, further studies are needed to assess the alteration of these mediators in response to treatment.

P1578
Effector and regulatory T cell subsets in hereditary hemorrhagic telangiectasia

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Purpose/Objective: Hereditary hemorrhagic telangiectasia (HHT) is a disorder characterized by recurrent epistaxis, cutaneous telangiectasia, and visceral arteriovenous malformations caused by mutations in the TGF-β receptor complex. Most cases are due to mutations in the *ENG* (endoglin) or *ACRLV1* genes. HHT patients have higher rates of severe extracellular bacterial infections, yet few studies have evaluated their immune functions. TGF-β plasmatic levels have been described as either increased or decreased in HHT patients and one study found a deficit on IFN-γ, TNF-α and IL-2 Th1 cytokines. As TGF-β has an important role on either regulatory T cells (Treg) and Th17 development, we investigated the levels of these two CD4 T subsets as well as Th1-polarized T cells in the peripheral blood of HHT patients.
Materials and methods: Sixteen HHT patients and 15 healthy donors (HD) were included. Treg on whole blood were identified by flow cytometry on a lymphocyte gate as CD3+CD4+CD25+CD127$^{low/neg}$ cells. Th1 and Th17-polarized T cells were identified after overnight culture of PBMC in serum-free AIM-V medium in the presence of PMA, Ionomycin and Brefeldine-A. Unstimulated cells were used as negative control. Cells were labeled with a viability amine-reactive dye, with antibodies to CD3, CD4 (surface) and to IFN-γ, TNF-α, IL-2 and IL-17 (after permeabilisation) to identify cytokine-producing T cells. Data was acquired on a Beckman Coulter flow-cytometer.
Results: Median numbers of Treg in HHT patients (9.1% of CD3+CD4+ cells, 5.3−13.7) were similar to Treg numbers in HD (7.9% of CD3+CD4+ cells, 6.7−10.9, $P = ns$). Also, no statistical differences were found for IL-17+ CD3 producing cells between HHT patients (0.65% of lymphocytes, 0.17−2.5) and HD (0.8% of lymphocytes, 0.2−3, $P = ns$). Also, there was no difference between HHT patients and controls concerning IFN-γ+ or TNF-α+: IFN-γ+CD3+ (HHT 23% of lymphocytes, 8−58 versus HD 15% of lymphocytes, 8−42, $P = ns$); TNF-α+ CD3+ (HHT 43% of lymphocytes, 20−73 versus HD 44% of lymphocytes, 29−58, $P = ns$). However, HHT patients had a significantly lower number of IL2+ CD3+ cells (35% of lymphocytes, 12−51) compared to HD (46% of lymphocytes, 18−54, $P < 0.05$, *t*-test).
Conclusions;Our results suggest that there are no major changes on effector or regulatory CD4 T cell subsets in HHT patients. Deficits on innate immunity (phagocytes) could explain increased rates of bacterial infections in this condition. Further studies with a greater number of patients could potentially favor the identification of eventual subtle changes on T cell function as well as segregation of data according to genetic mutations.

P1579
ELISpot but not ELISA detects human IL-21 secreted by antigen-stimulated human PBMC

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Purpose/Objective: IL-21 is primarily secreted by activated T cells, in particular CD4+ follicular and Th17 T cells, and regulates the development of T cells, B cells, NK cells and dendritic cells. Dysregulation of IL-21 has been reported to contribute to diseases such as SLE and RA. The fundamental relevance of IL-21 as well as its involvement in inflammatory and autoimmune diseases highlights the need for development and evaluation of suitable immunoassays facilitating analysis of IL-21.
Materials and methods: Monoclonal antibodies to human IL-21 were generated and used to develop sensitive capture ELISA and ELISpot assays. The assays were used to analyze IL-21 secretion by peripheral blood mononuclear cells (PBMC) after polyclonal (PHA) or specific stimulation (Tetanus toxoid; Candida albicans extract).
Results: Polyclonal activation of PBMC induced IL-21 production detectable by ELISpot and ELISA. Increasing PBMC concentrations and pro-longed incubation times increased the number of IL-21-secreting cells found by ELISPOT. IL-21 levels in supernatants, analyzed by ELISA, showed a reversed relationship with increasing cell concentrations and incubation times associated with a decrease in levels.

In PBMC activated with specific stimuli, IL-21-producing cells could be detected by ELISpot whereas IL-21 levels in supernatants were not measurable by ELISA. Similar results were obtained when analyzing IL-4 by ELISpot and ELISA, in parallel. IL-17 production after specific stimuli, on the other hand, was detectable both by ELISpot and ELISA.
Conclusions: Our data show that ELISpot can detect IL-21-producing PBMC under conditions where IL-21 levels in PBMC supernatants are not measurable by ELISA. Similar results were obtained with IL-4, a cytokine known to be difficult to detect by ELISA in supernatants from specifically activated PBMC due to consumption by cellular receptors. Difficulties to measure IL-21 by ELISA could thus depend on a low IL-21 secretion level per cell and/or receptor consumption. Further investigations of this aspect are ongoing. The results emphasize the importance of using suitable methods for analysis of IL-21.

Key words: human IL21; human IL17; human IL4; ELISpot; ELISA

P1580

Factors associated with parietal cell autoantibodies in the general population

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Purpose/Objective: The presence in serum of parietal cell autoantibodies (PCA) is a characteristic of autoimmune gastritis. We determined the prevalence of PCA in the general population and investigate their association with type 2 diabetes, insulin resistance and lifestyle factors related with autoimmune gastritis.
Materials and methods: A cross-sectional study was performed, involving 429 individuals enrolled in a cohort study of the general population of the Canary Islands. All participants underwent physical examination, provided a blood sample and responded to a questionnaire regarding health and lifestyle factors. Serum concentrations of PCA, soluble CD40 ligand (sCD40L), C-peptide and glucose (to determine insulin resistance) were measured. The association of PCA with the other factors was determined with bivariate analysis, and losgistic regression models were used to adjust the associations for age and sex.
Results: The prevalence of PCA was 7.8% (95% CI = 10.3−5.3). The factors associated with PCA were female sex ($P = 0.032$), insulin resistance ($P = 0.016$), menopause ($P = 0.029$) and sCD40L ($P = 0.019$). Alcohol consumption ($P = 0.006$) and smoking ($P = 0.005$) were associated with low prevalences of PCA. After adjustment for age and sex, the association with PCA was confirmed for smoking [OR = 0.1 (0.0−0.9)], alcohol consumption [OR = 0.3 (0.1−0.9)], insulin resistance [OR = 2.4 (1.1−4.9)], female sex [OR = 2.4 (1.1−5.3)], sCD40L [OR = 3.7 (1.2−11.4)] and menopause [OR = 5.3 (1.2−23.3)].
Conclusions: Smoking and alcohol consumption acted as protective factors against the appearance of PCA in the general population, whereas female sex, menopause, insulin resistance and elevated serum sCD40L were risk markers for PCA. In patients who smoke or drink alcohol, clinicians should be cautious when using PCA to rule out autoimmune gastritis.

P1581

Hematogonal cell populations in routine multiparametric flow cytometry analysis

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Purpose/Objective: Context: Multiparametric flow cytometry is a critical method in diagnosis and monitoring the evolution of neoplastic haematological processes, allowing quantitative classification, based on key molecular expression, of the cellular population structure in patient samples. Marker list selection and staining combinations are both crucial in defining normal and malignant proliferation, developmental and lineage cell identity, and may be useful to evaluate the minimal residual disease (MRD) and the postchemotherapy regenerating bone marrow.

Objective: To determine the feasability of using retrospective (repetitive) flow cytometry multiparametric data in documenting the MRD evolution and describing the regenerating haematological compartments.
Materials and methods: Repetitive bone marrow aspiration cell samples (six samples), covering a 6 year interval (2006−2011) of a B-acute lymphoblastic leukemia studied case (first diagnosed at age 15) were analysed using up to six color flowcytometry (FACS Calibur-CellQuest; FACS Canto II-FACS Diva). Staining protocol targeted with monoclonal antibodies the following molecules: CD45, CD19, CD22, CD20, CD10, CD34, CD38, TdT.
Results: Retrospective analysis of the six iterative bone marrow sample cell populations documents the complete therapeutical response of the tumor cell line (which was a phenotypically-homogenous B-cell precursor expressing CD19, CD10, CD34, CD22, intracellular CD79a and TdT), and, lately, the expansion of a B-cell precursor compartment that was heterogenous − and thus not evocative for relapse occurence (below 10%, CD45+ low CD19+ CD10+ CD34-/+, 0.6% CD22+ low CD20 s-/+, 3% CD38+ high).
Conclusions: Multiparametric flow cytometry can discriminate between regenerative postchemotherapeutical nature of cell expansions versus MRD or relapse.

P1583

Immune system genes expression in athletes with allergies

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Purpose/Objective: The aim of this study is to identify biomarkers for monitoring the functional state of the immune system in elite athletes.
Materials and methods: The expression of genes (mRNA) IL-1β, TNF-α, IL-8, IL18; TLR2, TLR4, TLR9, IFN-α, IFNγ, IL28, IL29, CD45, CD68, CD56, GATA3, TBX21, RORS2, Foxp3, TGF-β1 has been investigated. Quantitative mRNA was investigated by real-time PCR with reverse transcription and with normalization with respect to gene GUSB, HPRT1 B2M. We examined 22 athletes from the youth team of Russia on weightlifting. All the athletes were divided into three groups. Group 1 ($n = 10$) consisted of healthy athletes. Group 2 ($n = 7$) consisted of athletes who had chronic infection in anamnesis (frequent respiratory viral infection, chronic tonsillitis, herpes virus infection). Group 3 ($n = 5$) included athletes who had any manifestations of allergy in anamnesis and/or having a positive skin test and/or have elevated levels of total IgE in serum, but no allergy symptoms at the time of clinical examination. Epithelial cells scraping of the nasopharynx and peripheral blood mononuclear cells were used as a samples for the study of gene expression.
Results: The performed investigation demonstrated increased expression of genes involved in the inflammatory IL1, IL8 and innate immune receptors TLR2, TLR4, and reducing the expression of the transcription factor RORC2, providing feedback regulation of inflammation only in the scraping of the epithelial cells and only in athletes with allergies without exacerbation.
Conclusions: These results indicate that changes in immune function only in athletes with allergies (without exacerbation) in place of the primary immune cells contact with inhaled air, which may contain pathogens of respiratory infections and/or allergens.

P1584

Immunological features of IgA nephropathy development

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Purpose/Objective: IgA nephropathy (IgAN) is a major cause of end-stage kidney disease but at this moment there are no reliable prognostic criteria for the assessment of IgAN severity and none of the factors initiating this disease has been identified. Defects in systemic and mucosal immunity and immune complex clearance by mesangial cells (MCs) may be of importance in IgAN initiation.

The aim of this study was to estimate the contribution of immune cells and IgA clearance *via* IgA-binding receptors to IgAN development.

Materials and methods: MCs were isolated from nephrobiopsies of patients with IgAN ($n = 6$) and non-IgA-mesangioproliferative glomerulonephritis ($n = 4$) by incubation with 1 mg/ml collagenase I type and were characterized by vimentin$^+$ CD90$^+$44$^+$31$^-$14$^-$45$^-$ phenotype. Peripheral blood lymphocytes from patients with IgAN (34 ± 5 years, $n = 28$) and other glomerulopathies (31 ± 4 years, $n = 15$) were analyzed using CD3/CD5-PC7, CD4/CD8-PC5, CD19-PE, HLA DR/CD25-PE, Vγ2 TCR-Fitc, CD45RO-ECD monoclonal antibodies by means of flow cytometry FC500 (Beckman Coulter, USA). The level of serum immunoglobulins was determined by ELISA (VecktorBest, Russia).

Results: We have observed increased level of serum IgA only in 62% patients with IgAN, while the presence of IgA in the kidney mesangium was the basic diagnostic criterion of this disease. High level of IgA receptor – CD71 (81.2% (74.6% ÷ 85.4%) expression was detected in all MCs cultures. Other IgA-binding receptors (CD89, ASCRR) were not determined in MCs.

At the same time IgAN patients are characterized by significantly decreased percentage of $\gamma\delta$ T cells in peripheral blood compared with non-IgAN patients (1.8% (1.1% ÷ 2.7%) and 3.7% (1.8% ÷ 6.4%), respectively). Compared to patients with normal frequencies of CD8$^+$ T cells (30.3% (27.0% ÷ 33.9%); IgAN patients with CD8$^+$ T cells number exceeding 38% (40.2% (38.3% ÷ 41.4%) had decreased whole blood protein concentration, increased serum creatinine andsevere proteinuria (up to 2 g/day) ($P < 0.05$).

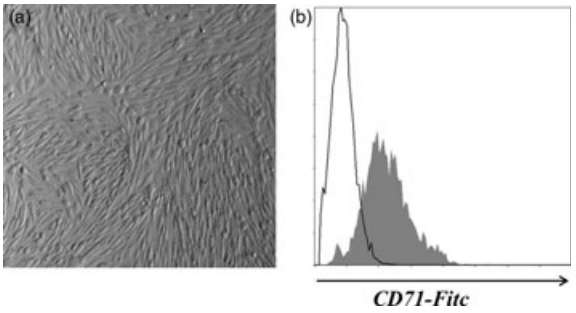

Figure 1. Morphology (A) and phenotype (B) of MCs culture.

Conclusions: The high level of CD71 expression on MCs of patients with various glomerulopathies is an evidence of the prevalence of immune system's abnormalities over kidney clearance in IgAN. We suggest that CD8$^+$ and $\gamma\delta$ T cells involve in IgAN development and progression and may be used as possible criteria for the assessment of this disease severity.

P1586

In vivo quantitative fluorescence imaging of matrix metalloproteinases activity in the CAIA mouse model of rheumatoid arthritis

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Purpose/Objective Matrix metalloproteinases including MMP-9 are known to be involved in diseases including inflammatory arthritis and contribute to pathological processes such as joint destruction. In RA patients MMP-9 is localized to sites of inflammation in synovium and is elevated in synovial fluid, serum and plasma (Ahrens *et al.*, 1996, Gruber *et al.*, 1996). Similarly in the collagen antibody induced arthritis (CAIA) mouse model MMP-9 mRNA levels are also elevated in the footpad (Chia *et al.*, 2008). The aim of this study was to determine *in vivo* MMPs activity at the site of inflammation at the acute and chronic stages of mouse CAIA using a fluorescence imaging techniques. In addition MMP-9 levels were measured in the serum.

Materials and methods: CAIA was induced in male Balb/c mice by an i.p. injection of 4 mg of monoclonal antibody cocktail to collagen II followed by 25 μg LPS 24 h later. 1 h prior to antibody cocktail administration mice were treated with 30 mg/kg Enbrel s.c. q.a.d.

The MMPSense 680, a fluorescent imaging agent, was used to detect *in vivo* activity of MMP-2, -3, -9 and -13 in hind paws at the peak of disease (d5) and in chronic stage (d12). Directly post imaging, serum samples were collected and MMP-9 concentration was measured using a mouse total MMP-9 immunoassay.

Results: Control mice showed first clinical signs of swelling 48 h post LPS challenge that peaked 72 h post challenge. The anti-TNF treatment showed significant inhibition on disease throughout the experiment.

The quantitative *in vivo* imaging showed significantly higher levels of MMP activity in inflamed paws compared to the negative control at acute and chronic stage. Positive control serum MMP-9 concentration was elevated compared to naïve serum in both, acute and chronic stages. The Enbrel treated mice showed a similar level of MMP activity as naïve mice at acute stage, however at the chronic stage MMP activity was significantly higher than naïve mice. This correlated with serum MMP-9 levels that were comparable to naïve levels at d5 and were significantly elevated in the chronic stage of disease. In addition the measured MMPs fluorescence correlated with clinical score in the chronic stage.

Conclusions: Activity of MMPs was significantly increased *in vivo* at site of inflammation during acute and chronic stage in the CAIA model. The localised MMP activity correlated with measured MMP-9 concentration in serum from all treatment groups. Moreover the MMP activity could be inhibited by anti-TNF treatment in this model. This indicates that MMP-9 is a valuable biomarker for disease progression in CAIA model and could be used as an addition to clinical score.

P1588

LC/DAD analysis of serum biogenic amines in patients with diabetes mellitus, chronic urticaria and Hashimoto's thyroiditis

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Purpose/Objective: The biogenic amines putrescine (Put), histamine (His), spermidine (Spd), *N*-acetyl putrescine (NAP), *N*-acetyl spermidine (NAS), dopamine (Dop), epinephrine (Epi), norepinephrine (NE) and spermine (Spm) are a group of naturally occurring compounds exerting a large number of biological effects.

This study was commenced to elucidate the role of biogenic amines as possible diagnostic markers for three autoimmune diseases: *Diabetes mellitus*, *Chronic urticaria*, and *Hashimoto's thyroiditis*.

Materials and methods: This study involved 20 patients with *Diabetes mellitus*, 20 patients with *Chronic urticaria*, eight patients with Hashimoto's thyroiditis, and 20 healthy volunteers. We precipitated serum proteins using 0.4 M $HClO_4$. At pH 8.0 we performed derivatization with dansyl-chloride. 50 μl of prepared serum samples were injected into LC/DAD, in conditions of gradient elution, on C18 column. Commercially available Put, His, Spd, NAP, NAS, Dop, Epi, NE, and Spm were dissolved in different concentrations in ultra pure water; treated in the same way as serum samples and injected into LC. Calibration curves were made by plotting peak area values against the respective concentrations of standards. The qualitative analysis was done using the method of retention time, and quantitative analysis using external calibration. The recovery study was carried out using real serum sample from healthy control, by spiking techniques.

Results: Retention times were 6.6 min for NAS, 8.8 min for NAP, 9.1 min for Put, 10.1 min for His, 13.2 min for Spd, 13.9 min for NE, 14.7 min for Epi, 14.9 min for Dop, and 15.4 min for Spm, respectively. Obtained data showed excellent linearity of calibration curves for Put, His, Spd, NAP, NAS, Dop, Epi, NE, and Spm. Compared to controls, His levels in *Diabetes mellitus* patients were statistically higher; in *Chronic urticaria* patients levels of Put and His were lower; Spd, NE and Epi levels were enhanced; Put was statistically lower and Spd higher in *Hashimoto's thyroiditis* patients. *Chronic urticaria* patients were the only in whose serum NE was found. NAP, NAS, Dop, and Spm were under limits of detection.

Conclusions: Preliminary results from this study showed diverse distribution of investigated biogenic amines indicating different activation of metabolic pathways controlling biogenic amine biosynthesis and degradations in analyzed autoimmune diseases.

P1589

Levels of inflammatory mediators in gingival crevicular fluid from patients with periodontal disease before and after treatment

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Purpose/Objective: The purpose of the present research was to determine the levels of IL-1α, IL-1β, TNF-α, MMP-3 and MMP-8 in gingival crevicular fluid (GCF) of subjects with chronic periodontitis before and after non surgical treatment.

Materials and methods: Clinical measurements were carried out in 11 patients diagnosed with chronic periodontitis and 11 periodontally healthy controls. The clinical indexes evaluated were: gingival index (GI), plaque index (PI), bleeding on probing (BOP), probing depth (PD) and attachment loss (AL). The measurements were taken at six sites per tooth in all teeth in each subject. GCF samples were taken from one tooth per quadrant, and the levels of these mediators were measured using an ELISA test.

Results: A statistically significant difference ($P < 0.05$) was observed in all the clinical parameters between patients and control group before and after periodontal treatment. Correlations between levels of mediators with the clinical parameters were not observed. Statistically significant differences were found between patients and control group in relation with levels of inflammatory mediators ($P < 0.05$) before and after treatment. The levels of IL-1α, IL1-β, TNF-α, MMP-3 and MMP-8 decreased in 44.2%, 48.35%, 53.28%, 62.16% y 34.03% respectively.

Conclusions: Periodontal therapy reduced the levels of the inflammatory mediators evaluated in this study, which were significantly associated with the severity of periodontal disease. This research was supported by CDCH PG: 10-00-7070-2007.

P1590

Measuring autoantibodies against IL-17F and IL-22 in autoimmune polyedocrine sydnrome type I

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Purpose/Objective: Patients with autoimmune polyendocrine syndrome type I (APS I) have at least two of the three disease components adrenal insufficiency, hypoparathyroidism and chronic mucocutaneous candidiasis. Various other organ-specific autoimmune manifestations are common, as well as a number of ectodermal symptoms. The underlying cause of APS I is mutations in the gene encoding the autoimmune regulator protein AIRE. Deficiency of this protein leads to loss of immunologic tolerance and release of autoreactive T-cells from thymus into the periphery.

Patients frequently develop high titers of autoantibodies against molecular targets in their affected organs. The pathological role of these antibodies is unknown, but they have become important markers for APS I and other autoimmune conditions. Autoantibodies against interleukin (IL) -17A, IL-17F and IL-22 have recently been described in patients with APS I, and their presence is reported to be highly correlated to chronic mucocutaneous candidiasis (CMC). The aim of this study was to develop a robust high-throughput radioligand binding assays (RLBA) measuring IL-17F and IL-22 antibodies, and to compare them with current enzyme-linked immunosorbent assays (ELISA) of IL-17F and IL-22; moreover to correlate the presence of these antibodies to the presence of CMC.

Materials and methods: A total of five RLBAs were developed based on IL-17F and IL-22 monomers and homo- or hetero dimers. As these

interleukins are small molecules, hence difficult to express *in vitro*, they were fused as dimers, which proved to increase the efficiency of expression. Sera from 25 APS-I patients were analysed by the different RLBAs and ELISA.
Results: Analysing the presence of these autoantibodies in 25 Norwegian APS I-patients revealed that the different RLBAs detected anti-IL-17F and anti-IL-22 with high specificity, using both homo- and heterodimers. The RLBAs based on dimer proteins are highly reproducible with low inter- and intra-variation.
Conclusions: The RLBAs have the advantages of high throughput and easily standardisation compared to ELISA, thus proving excellent choices for the screening of IL-17F and IL-22 autoantibodies.

P1594
Preanalytical and analytical factors affecting assessment of extracellular vesicles as biomarkers

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Purpose/Objective: Current research in the field of extracellular vesicles (including exosomes, microvesicles and apoptotic bodies) is undergoing a revolutionary development. Extracellular vesicles are considered both as disease-specific biomarkers and therapeutic vehicles. Our goal was to identify preanalytical and analytical factors that may have an impact on the detection of cell-derived extracellular vesicles.
Materials and methods: We tested the effect of different anticoagulant tubes, shaking, storage at different temperatures, centrifugation forces and times and the effect of size filtration on the number and structure of blood plasma derived extracellular vesicles assessed by flow cytometry, transmission electron microscopy, atomic force microscopy, dynamic light scattering and qNANO techniques.
Results: Our data indicate that the number of platelet derived microvesicles is strongly influenced by the type of anticoagulation. Shaking and storage of blood plasma samples at 37°C proved to be strong inducers of platelet derived microvesicles. Forced filtration caused fragmentation of extracellular vesicles. Furthermore, we found that protein aggregates (such as immune complexes, biotin-avidin complexes) shared biophysical parameters with microvesicles and resulted in microvesicle-mimicking signals during flow cytometry. We developed a differential detergent lysis method to differentiate microvesicles from protein aggregates. Here we show that by using our improved method of microvesicle assessment, important new insights are gained into the pathomechanism of diseases such as rheumatoid arthritis.
Conclusions: Correct assessment of extracellular vesicles in biological fluids requires standardization of both preanalytical and analytical parameters.

P1595
Preoperative serum levels of CA 242 in colorectal carcinoma

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Purpose/Objective: Biochemical markers for colorectal carcinoma (CRC) are potentially useful in screening for early disease, aid in diagnosis, determining prognosis, surveillance of patients undergoing curative resection and monitoring the treatment of advanced disease.
Materials and methods: Preoperative serum levels of CA242was determined using ELISA technique for 35 patients with colorectal carcinoma, 25 patients with benign colorectal diseases (ulcerative colitis) and 10 volunteers as control group.
Results: CA242 showed to be high distributed in patients with colorectal carcinoma which represented 37.1% as compared to patients with ulcerative colitis and control group which represented (16% and 0%) respectively ($P = 0.026$). The overall sensitivity of the CA242 test was 37.1% and the corresponding specificity was88.6%, using cut-off level 20 U/ml for CA 242. In regard to effect of age on the level of CA242 in study groups, it was showed to be high distributed in young age from (36 to 65) years which represented 17.1% ($P = 0.088$). Regarding to gender, CA242 showed to be high distributed in females which represented 61.5% as compared to males which represented 38.5% ($P = 0.149$). In regard to effect of grade (degree of differentiation) on the level of CA242, it showed to be high distributed in poorly differentiated colorectal adenocarcinoma which represented 38.5%, while in well and moderately differentiated colorectal adenocarcinoma which represented (30.7% and 15.4%) respectively ($P = 0.588$). When the stage of disease takes in a consideration CA 242 was 7.7%, 7.7%, 15.4% and 38.5% in stage (I, IIA, IIB, IIIB and IV) respectively ($P = 0.218$).
Conclusions: In conclusion these findings suggest that CA242 elevated level and high sensitive in patients with colorectal carcinoma and may be used as an indicator of disease activity and may be considered as prognostic factor for patients with colorectal carcinoma. CA242 high distribution in young age may be attributed to incidence of colorectal carcinoma in Iraq occur in young age, but the cause of why was CA242 in females higher than males remain unclear. In patients with ulcerative colitis, CA242 may be used as predicting factor for malignant transformation.

P1596
Serum ghrelin and adiponectin levels are increased in underweight COPD patients, but leptin and proinflammatory cytokines are not changed

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Purpose/Objective: Weight loss, muscle wasting, and tissue depletion are common features reported in COPD patients and they are all related with systemic inflammation. We investigated the relations between pulmonary functions, circulating levels of inflammatory mediators and metabolic parameters in underweight COPD patients.
Materials and methods: Biochemical, hormonal, and anthropometric parameters, serum levels of adiponectin (ApN), ghrelin, leptin, hsCRP, IL-6, IL-1b, IL-8, TNF-α and pulmonary functions were evaluated. COPD patients were grouped according to Global Initiative for Chronic Obstructive Lung Disease criterion. Patients and control subjects were all male. Group 1: Mild-moderate COPD patients ($n = 18$; with a mean age of 66.4 ± 9.2 years; range 45–79 years and body mass index (BMI): 19.7 ± 1.5 kg/m^2), group 2: Severe-very severe COPD patients ($n = 32$; with a mean age of 65.9 ± 10.0 years; range 45–80 years; BMI: 19.3 ± 1.6 kg/m^2), group 3: Control group was composed of healthy subjects with normal BMI and pulmonary function tests ($n = 17$; with a mean age of 50.2 ± 8.4 years; BMI: 21.85 ± 1.5 kg/m^2). The mean duration of COPD was 7.1 ± 6.4 years for group 1 and 7.3 ± 5.1 years for group 2. All patients were clinically

stable for at least 3 months and were not smoking for at least 6 months.

Results: ApN concentration was higher in group 1 (43.3 ± 28.6 ng/ml; $P < 0.05$) and group 2 (59.9 ± 31.8 ng/ml; $P < 0.001$) when compared with control group (23.5 ± 13.6 ng/ml). Ghrelin concentrations were higher in both COPD groups (1281.0 ± 1173.7 and 1840.0 ± 403.6 pg/ml; $P < 0.05$) than the controls (554.0 ± 281.9 pg/ml). No significant increase for leptin, IL-1b, TNF-α, IL-8 was found between groups. IL-6, hsCRP levels were higher in group 1 than control group. ApN was negatively correlated with BMI and FEV1. Skinfold thickness were decreased in COPD patients when compared with control group.

In the whole group, FEV1 was positively correlated with BMI, skinfold thicknesses of biceps, triceps and abdomen, insulin, tryglyceride whereas was negatively correlated with age, pack/years, HDL-Chol and ApN. We detected increased SHBG level with decreased insulin and HOMA-IR which mayindicate increased insulin sensitivity.

Conclusions: We can conclude that antiinflammatory effect of ApN and ghrelin had been more evidentin severe-very severe COPD patients.

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P1597

Significance of T helper 17 immunity in hepatitis C virus recurrence in orthotopic liver transplantation

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Purpose/Objective: Hepatitis C virus (HCV) recurrence is universal following orthotopic liver transplantation (OLT) with an accelerated rate of fibrosis in the allograft compared to that in the native liver in chronic HCV. CD4+ T cells play an important role in HCV immunity and T-helper type 17 (Th17) cells have also been implicated in inflammatory proccesses associated with liver fibrosis. Our aim was to determinate the role of CD4+ Th17 cells in HCV recurrence in liver allograft transplantation.

Materials and methods: Thirty OLTr and eighty four healthy donors (HD) from Murcia Region, Spain was included in this study. To quantify the frequency of CD4+ Th17 cells, the OLTr and HD bloods was cultured during 4 h in humidified 5% CO_2 at 37°C and used for an intracytoplasmatic flow cytometry assay. To measure IL-17 cytokine levels, we used a specific ELISA test (Quantikine® Human IL-17 Immunoassay R&D System) after cultured the OLTr and HD bloods with Concanavaline A (ConA-Sigma®). Statistical analysis was performed by using SPSS vs15.0 for Windows (SPSS, Chicago, IL). Mann-Whitney test was used to determinate significant differences in the frecuency of CD4+ Th17 cells or IL-17 cytokine levels between three groups (AR = Acute Rejection, NAR = Non-Acute Rejection and Control). Two-sided level of significance was set at $P < 0.05$.

Results: We analyzed the frequency of CD4+ Th17 cells in non-HCV recurrence group, HCV recurrence and controls. The frequency of CD4+ Th17 cells in patients with HCV-recurrence increase since the beginning of orthotopic liver transplantation in comparison to NHCV group. Moreover IL-17 cytokine levels were higher in patiens with HCV-recurrence than in NHCV in basal situation.

Conclusions: This data suggest that the CD4+ Th17 cells plays an important role in HCV immunity.

P1598

Skin immunity in complex regional pain syndrome: epidermal langerhans cell density is significantly different between CRPS and non-CRPS affected limbs

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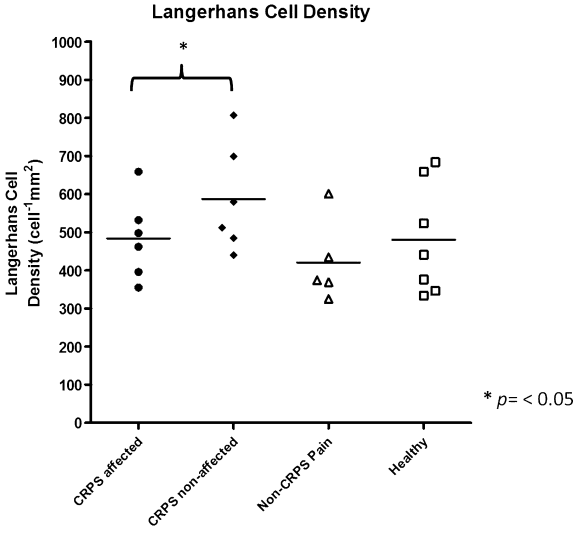
Purpose/Objective: We are investigating the role of the immune system within Complex Regional Pain Syndrome (CRPS) affected tissues in order to elucidate any possible role for immune cells in the generation or maintenance of chronic pain. Currently we are focused on cutaneous immunity and in particular on epidermal dendritic cells known as Langerhans cells (LCs). We hope that by characterizing any differences in immune cell infiltrate, phenotype, and function within CRPS limbs we can further understand the pathophysiology of the disease and discover new diagnostic indicators or avenues for therapeutic intervention.

Materials and methods: Two 6mm skin punch biopsies were taken from CRPS patients ($n = 6$), one from a CRPS affected limb and another from a non-CRPS affected contra-lateral control limb. Biopsies were then incubated in 2 mM EDTA for a minimum of 2 h before separating the dermis and epidermis. Epidermal sheets were then stained for LCs in a two step procedure using the cell specific antibody CD1a. Co-staining with HLA-DR and appropriate controls were also performed. Epidermal sheets were imaged using 3 dimensional confocal microscopy and LC densities calculated as cells/mm^2 of epidermis.

Results: LC density in CRPS affected limbs was 484 ± 44 cells/m^2 and in CRPS non-affected limbs was 587 ± 57 cells/mm^2. These values were found to be significantly different using a paired T-test analysis ($P = <0.05$). There was no significant difference between healthy control limbs and CRPS affected or non-affected limbs owing to significant donor variation.



Conclusions: Altered LC density further implicates the immune system in the pathogenesis and maintenance of CRPS. LCs are also regulated by sympathetic nerves and so can act as a synapse between the nervous and immune systems, a position which has obvious relevance in CRPS. It is not known at this stage if altered LC density

plays a functional role in CRPS or is a reliable indicator of disease. Research is ongoing in this area to fully elucidate the significance of this finding.

P1599

Soluble molecule profiling and network analysis of primary Sjögren's syndrome patient serum

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Purpose/Objective: In this study, we examine the serum samples of a large cohort of clinically well-characterised PSS patients and healthy controls in order to determine whether serum cytokines and chemokines may be used to differentiate PSS patients from healthy controls, and if so, the relationship between these serum abnormalities and clinical phenotypes.
Materials and methods: Serum levels of 24 different cytokines, chemokines and adhesion molecules for 150 pSS patients and 30 healthy controls were measured using Cytometric Bead Array (BD Biosciences). PSS patients were further classified into the following subsets: (1) Lymphoma/paraprotein positive; (2) High systemic disease activity; (3) Low residual glandular function; (4) High Fatigue. The relationship between the levels of each analyte and clinical and laboratory parameters of PSS was examined using multivariate analysis and Mann-Whitney *U* testing. Complex network analysis was performed using Cytoscape, where interactions can be integrated with and validated using published data.
Results: There were marked differences in the levels of many cytokines and chemokines between PSS patients and healthy controls, with a P value < 0.001, statistically significant after Bonforroni's correction for multiple comparisons. Serum IL4 and IL17 were found to be significantly higher in patients versus controls. Chemokines such as MIG (CXCL9), MIP1a (CCL3) and MIP1b (CCL4), IP10 (CXCL10), were also measured at higher levels in patient serum. IFNa, LTA and TNFa serum levels differ significantly between patients and controls. Network analysis has helped provide extra insight into the complex interactions between the studied serum analytes.
Conclusions: Differences in blood cytokine and chemokine levels between primary Sjogren's patients and controls can be detected in serum through the use of Cytometric Bead Arrays. Serum MIG (CXCL9), MIP1a (CCL3) and MIP1b (CCL4), IP10 (CXCL10) IFNa, LTA and TNFa levels differ significantly between patients and controls. Our observations raise the possibility that these analytes may be important in disease pathogenesis.

P1603

The role of T helper 17 cells in acute allograft rejection development in liver transplantation

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Purpose/Objective: Orthotopic Liver Transplantation (OLT) is currently accepted as a viable therapeutic option for various end-stage liver disease and the incidence of acute rejection after OLT was still more than 30% where CD4+ T lymphocytes have been implicated in playing critical roles in allograft rejection. Recently, several groups have reported that under allograft rejection conditions OLT recipients (OLTr) showed a higher levels of IL-17 cytokine with a higher frequency of CD4+ Th17 cells. The aim of our study was determinate the role of CD4+ T helper 17 cells in OLTr either with and without acute rejection.
Materials and methods: Thirty OLTr and eighty four healthy donors (HD) from Murcia Region, Spain was included in this study. To quantify the frequency of CD4+ Th17 cells, the OLTr and HD bloods was cultured during 4 h in humidified 5% CO_2 at 37°C and used for an intracytoplasmatic flow cytometry assay. To measure IL-17 cytokine levels, we used a specific ELISA test (Quantikine® Human IL-17 Immunoassay R&D System) after cultured the OLTr and HD bloods with Concanavaline A (ConA-Sigma®). Statistical analysis was performed by using SPSS vs15.0 for Windows (SPSS, Chicago, IL, USA). Mann-Whitney test was used to determinate significant differences in the frecuency of CD4+ Th17 cells or IL-17 cytokine levels between three groups (AR, Acute Rejection; NAR, Non-Acute Rejection and Control). Two-sided level of significance was set at $P < 0.05$.
Results: We analyzed CD4+ T helper 17 cells frequency between AR, NAR and CONTROL groups and we have seen that the frequency of CD4+ T helper 17 cells in AR group before OLT was higher than CONTROL group, furthermore IL-17 interleukine cell culture supernates levels was higher in AR group in the firsts fifteen days after transplantation in comparition to NAR. In this study, we have found an inter-relationship between CD4+ Th17 cells frequency and IL-17 cytokine levels with acute rejection development in a OLTr cohort.
Conclusions: More researchers have been reported that serum levels of IL-17 were increased in patients with acute hepatic rejection which is consistent with our results in liver transplantation. In this study, we provide evidence that Th17 cells participates in the allogeneic liver rejection following liver transplantation, however the mechanism by which Th17 cells promotes allograft rejection in the liver remains to be determinated.

P1604

Tissue bank for inflammatory diseases Heidelberg/Gewebebank für entzündliche Erkrankungen Heidelberg (GEZEH): an innovative platform for translational immunology

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Purpose/Objective: The tissue bank for inflammatory diseases (GEZEH) in Heidelberg, Germany, was founded in 2011 by the Institute of Pathology and the Department of Dermatology as a section of the Sonderforschungsbereich 938. It is a nonprofit organization with a completely evaluated legal and ethical framework and is embedded in the Biomaterial Bank Heidelberg (BMBH) concept. Its main aim is the acquisition and characterization of fresh-frozen and paraffin-embedded non-neoplastic human tissues according to the standards of good scientific practice and the promotion of interdisciplinary translational immunology research of the Sonderforschungsbereich and its cooperating partners.
Materials and methods: It also offers expert project assistance: a project leader can submit a short proposal, and the tissue collecting/preparing process will be performed in cooperation with a specialised pathologist and, if applicable, an experienced clinical researcher.
Results: The tissue bank, in cooperation with the tissue bank of the National Centre for Tumour Diseases (NCT) in Heidelberg is also a central platform for further developing of innovative technologies for tissue handling, e.g. multi-tissue-array and virtual microscopy, with links to digital image analysis and bioinformatics.

Conclusions: Thus, the GEZEH tissue bank represents a model for innovative biobanking and for institutions with active interdisciplinary translational immunology research.

P1605

Use of biomarkers in milk for monitoring the systemic health status in Holstein dairy cows

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Purpose/Objective: During peripartum immunosuppression and early lactation, dairy cows show increased susceptibility for local and systemic infections. These infections impair animal health and the economic efficiency of dairy farms. Thus, long-term maintenance of health is a basic requirement for successful herd management. The objective of this study is to detect immunological markers in milk which are indicative of the overall health status and to develop an immunoassay for marker analysis in milk. Ultimately, we aim to provide an on-farm testing technology for the monitoring of herd health and estimation of the breeding value.
Materials and methods: Blood and milk samples of Holstein cows in various health conditions (healthy/no apparent infection, mastitis, extra-mammary disorder) were collected in co-operation with local dairy farms. Blood leukocyte and milk somatic cell (MSC) populations were analyzed by flow cytometry. RNA from MSC of cows with clinical mastitis (CNS, $n = 2$; *S. uberis*, $n = 4$), endometritis (score 1: $n = 4$; score 2: $n = 2$) and matched healthy controls (2*4 years, 1st or 2nd lactation, 10*100 d pp) was applied to a bovine genome Array (Affymetrix®) for transcriptome analysis. Results were validated by analyzing selected differentially expressed genes by quantitative RT-PCR and corresponding proteins by ELISA.
Results: Cows with infectious diseases had higher percentages of $CD11b^+$ granulocytes in blood and mammary gland secretions, whereas in milk from healthy cows, lymphocytes, especially $CD8^+$ T lymphocytes and epithelial cells, were the predominant cell populations. In both milk and blood, a $CD25^+/CD11b^+$ cell population was present during infections and disorders. Transcriptome analysis revealed activation of innate immune functions (i.e. TLR pathways, chemokine signalling) and differential expression of acute-phase genes (i.e. Lactoferrin, Pentraxin 3, Haptoglobin) particularly during mastitis. Furthermore, IL2RA was up-regulated during infection, confirming results from flow cytometric analysis (CD25). In particular, acute-phase proteins in milk showed discriminatory ability for mastitis and extra-mammary infections or disorders when analyzed by ELISA.
Conclusions: Our results suggest a panel of immunological markers in milk that can be used to evaluate the systemic health status of dairy herds and will be validated in further studies.

P1606

Which anti-coagulant should I use?

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Purpose/Objective: Blood for research purposes is often collected into an anticoagulant. Different anticoagulants are available and they typically interrupt the coagulation cascade by chelating calcium or inhibiting thrombin. Each anticoagulant might differentially affect cells within blood so the appropriate anticoagulant must be chosen. This study aimed to determine the effect of four anticoagulants on cell types within adult peripheral blood.
Materials and methods: Blood was taken into heparin, sodium citrate (SC), acid citrate dextrose (ACD), or ethylenediaminetetraacetic acid (EDTA). Full haematology counts and differentials were taken. Whole blood cultures were stimulated with LPS (10 ng/ml) and PHA (5 μg/ml) for 24 and 48 h, respectively. Mononuclear cells (MNC) were prepared by density gradient centrifugation and cultured under the same conditions. Cytokine levels (IL-6, IL-1β and IL-17A) in the culture supernatants were analysed.
Results: There was no significant difference between total and differential cell counts for each of the four anticoagulants although a correction for dilution by anticoagulant must be used for ACD and SC. For whole blood cultures, LPS and PHA-induced cytokine production was significantly lower in blood collected into ACD (IL-6 $P = 0.002$; IL-1β $P = 0.019$; IL-17A $P = 0.047$), SC (IL-6 $P = 0.008$; IL-1β $P = 0.015$) and EDTA (IL-6 $P = 0.002$; IL-1β $P = 0.021$; IL-17A $P = 0.044$) compared with blood collected into heparin. The choice of anticoagulant had no significant effect on cytokine levels in MNC cultures.
Conclusions: The choice of anticoagulant is an important component of experiment design; if cellular analyses are to be on whole blood then an anti-coagulant that does not chelate calcium should be chosen. As the anticoagulant is likely washed away during preparation of MNCs the choice of anticoagulant is less critical. Further investigation is needed to understand the influence of anticoagulants on cell activation.

Poster Session: Cell Therapy

P1607

4-1BB engineered human T-cells demonstrate enhanced function and mediate regression of established melanoma tumors

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Purpose/Objective: Gene modification of T-cells for cancer immunotherapy has been shown to mediate the regression of large solid tumors in cancer patients. However, inadequate T-cell persistence and reduced reactivity may represent major factors that would limit the clinical response to those treatments. Co-stimulation can actively shape the lymphocyte response. 4-1BB (CD137) is a co-stimulatory molecule expressed on T cell surface following TCR engagement, while its ligand 4-1BBL can be found on professional antigen presenting cells, but more importantly, also on the surface of tumor cells. The role of 4-1BB/4-1BBL pathway has emerged as central in cytotoxic CD8+ responses and survival, and consequently, it is possible that its manipulation could provide us with a way to change current anti-tumor cellular therapies.
Materials and methods: Herein, we generated a producer-cell clone which retroviral supernatant yielded high levels of transduction and constitutive expression of 4-1BB in human lymphocytes and tested the anti-tumor function of 4-1BB modified T-cells.
Results: 4-1BB-transduced T-cells produced from healthy donors or from vaccinated-melanoma patients exhibited enhanced cytokine secretion and upregulation of activation markers upon co-culture with melanoma tumor lines. In addition, these cells were able to expand and proliferate at a higher rate and expressed higher levels of the anti-apoptotic molecule Bcl-xL, compared to control cells. These cells were also rather insensitive to immunosuppression mediated by TGFβ. Finally, we tested the function of these cells in a CAM-based model of human melanoma tumors and we showed that 4-1BB-engineered human T-cells can mediate regression of established tumors.
Conclusions: Overall, we conclude that the modification of human T-cells with 4-1BB yields improved anti-tumor function which may apply to the genetic modification of cancer patient lymphocytes.

P1608

Abrogation with AB serum of TCR-independent streptamer and pentamer binding interactions: improving the identification of virus-specific CD8+ cells by multimer technology

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Purpose/Objective: Multimeric MHC class I-peptide complex-based methodologies have shown promising results in the identification and selection of antigen-specific cytotoxic ($CD8^+$) T-lymphocytes.

Non-specific interaction of multimers to $CD8^-$ cells has been observed by several authors, overestimating specific-T cell quantification and contaminating the selected therapeutic cellular product by un-desired cellular lineages.

The aim of this study was to assess the use of human AB (HAB) serum as a method for blocking the non-specific binding of Pentamer (PM) and Streptamer (ST) multimers.
Materials and methods: *FcR blockade with human AB serum*: Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of 20 HLA-A*0201 healthy donors seropositive for CMV. 1×10^6 cells were incubated with HAB serum for 10 min at room temperature (RT).

Multimer and monoclonal antibody (mAb) staining: 0.75 mg PE-labelled Strep-Tactin and 0.5 mg HLA-A*0201/STpp65$_{495-503}$ were mixed. 0.2 mg ST complex and 5 ml of HLA-A*0201/PMpp65$_{495-503}$ were incubated with PBMC for 45 min at 4°C and 10 min at RT, respectively. After multimeric staining, anti-human pan-T, CD8, CD14 and CD19 mAb were added. 500000 cells were acquired in a FACSCanto II equipment. Wilcoxon signed-rank and Mann-Whitney tests were used for statistical analysis.
Results: Multimer-positive cells are likely to represent cross-contamination by cells lacking a pp65-specific TCR, as the detected median frequency of total PM/ST-positive cells is higher than the frequency of CMV-specific $CD8^+$ cells. The cells that bind non-specifically to multimers have been identified as $CD19^+$ and $CD14^+$.

After the blockade with HAB serum, we have observed that the frequency of CMV-specific $CD8^+$ cells remains stable, while the overall frequency of multimer-positive cells was reduced significantly ($P = 0.035$ for PM and $P = 0.004$ for ST). A significant reduction of the frequency of multimer-positive $CD8^-$ cells ($P = 0.045$ for PM and $P < 0.01$ for ST) was also observed. Thus, the ability of HAB to significantly reduce non-specific binding to PM is similar to that of ST ($Z = -0.89$, $P = 0.371$).
Conclusions: TCR-independent unwanted binding between multimeric complexes and non $CD8^+$ cells can be efficiently suppressed by the incubation of the PBMCs with HAB serum.

P1610

Adoptive transfer of immature dendritic cells prevents progression of established atherosclerosis in LDLr KO mice

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Purpose/Objective: Dendritic cells (DCs) have the unique capacity to both initiate and regulate the immune response. Due to these properties, DCs have been used as immunotherapy in many different models for autoimmune diseases. In the present study we explored the possibility to use immature DCs (ImDCs) as a therapy in a mouse model for established atherosclerosis.
Materials and methods: Male LDL receptor knockout mice were put on a Western-type diet (WTD) for 20 weeks to induce established atherosclerosis. At this time point the baseline group was sacrificed. Remaining mice received 3 i.v. injections ImDCs or PBS (1.5×10^6 cells in 100 μl PBS) and were kept on WTD for an additional 10 weeks. After a total of 30 weeks on diet, mice were sacrificed.
Results: Plaque area analysis showed that treatment with ImDCs prevented lesion progression at major sites throughout the vasculature. We hypothesized that high cholesterol levels present in the circulation of LDL receptor knockout mice could alter the status of the DCs into a tolerogenic phenotype. Indeed, a tolerogenic semi-mature phenotype was observed *in vitro* after cholesterol loading of ImDCs in the presence of hypercholesterolemic mouse serum and lipoproteins isolated from hypercholesteremic serum. More importantly, loading of ImDCs with lipoproteins reduced the capacity to induce antigen-specific proliferation. Also *in vivo*, a semi-mature tolerogenic phenotype was observed after the adoptive transfer of GFP^+ ImDCs into hypercholestermic LDL receptor knockout mice. Furthermore, treatment with ImDCs increased Foxp3 and TGF-β mRNA expression in

lymph nodes and increased the number of atheroprotective regulatory T cells in blood and lymph nodes. Finally, treatment lowered the lesional inflammatory status as indicated by decreased $CD3^+$ T cell infiltration and reduced necrotic core size.
Conclusions: Cholesterol loading of ImDCs induces a semi-mature tolerogenic phenotype both *in vitro* and *in vivo* with reduced capacity to induce specific T cell responses. These tolerogenic DCs exert atheroprotective functions leading to prevention of plaque progression in a model for established atherosclerosis.

P1611
Amelioration of ulcerative colitis and colorectal cancer by adoptive transfer of antigen-redirected regulatory T-cells

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Purpose/Objective: The goal of this study was to examine whether antigen-redirected regulatory T-cells (Tregs) can be engineered to suppress ulcerative colitis and its associated colorectal tumors. To this end we combined the advantages of the T-body approach, which endows T-cells with antibody-type specificity, with the immunosuppressive activity of Tregs. Based on the elevated carcinoembryonic antigen (CEA) levels in the epithelia of colitis patients, we employed a model system of CEA transgenic mice and demonstrated the therapeutic potential of CEA-specific Treg T-bodies.
Materials and methods: A CEA-specific chimeric antigen receptor (CAR) was introduced *ex vivo* into isolated naïve Tregs by retroviral transduction. The CEA-specific Treg T-bodies were then adoptively-transferred into CEA transgenic mice inflicted with colitis. Two disease models were employed: the azoxymethane-dextran sodium sulfate (AOM-DSS) model for colitis and colorectal cancer, as well as T-cell induced colitis model. Colitis severity and tumor progression were monitored by endoscopic examination in live animals.
Results: Mice treated with CEA-specific Treg T-bodies showed a marked reduction in colitis severity, this in comparison to mice treated with control Treg T-bodies of irrelevant specificity. Moreover, in the AOM-DSS model CEA-specific Treg T-bodies significantly decreased the subsequent colorectal tumor burden.
Conclusions: CEA-specific Treg T-bodies showed a promising potential in ameliorating ulcerative colitis and in hindering tumor development in mice.

P1612
Analysis of Ly6ChighCD11b+ monocytes generated *in vitro* in inflammatory animal models

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Purpose/Objective: Circulating monocytes provide defense against infections and also contribute to autoimmune diseases. Two types of blood monocytes were recently identified in mice. Ly-$6C^{low}CD11b^+$ phenotype migrate to uninjured tissues and differentiate into resident macrophages and dendritic cells (DCs). In contrast, a distinct inflamed monocyte subset with a Ly-$6C^{high}CD11b^+$ phenotype infiltrates infected tissue and contributes to the development of inflammation. Ly-$6C^{hi}$ monocytes, the aim of our work, represent 2–5% of whole monocytes presents in the bloodstream of mice. Since the Ly-$6C^{high}CD11b^+$ monocytes, are difficult to obtain, from mouse blood and the amount obtained is very low, our group developed a novel system to generate them *in vitro* from mice bone marrow.

Our goal in this study is to determine the function *in vivo* of Ly-$6C^{high}CD11b^+$ monocytes, in two animal models of inflammation.

Materials and methods: Cells were sorted in order to obtain Ly-$6C^{high}CD11b^+$ monocytes that display the phenotype as described in cells obtained from the blood flow, Ly-$6C^+$, $CD62L^+$, $CCR2^+$, $CD11b^+$, $F4/80^+$. These cells could be activated *in vitro* producing M1 or M2 cytokines when activated with IFN-γ or IL-4, respectively.

The following step was to inject these sorted cells intra venous (i.v) in both animal models. To analyze the migration capacity, we used two different animal models to induce local inflammation in non-sensitizied, immunocompetent Balb/c mice. In the first model, 1-fluoro-2,4-dinitrobenzene (DNFB) was applied topically on the mouse right ear to create skin-homing conditions (DNFB skin model), in the second model, an injection of Notexin was applied into the anterior tibialis (AT) on the mouse right leg to induce myoinjury (Notexin model). This toxin can lead to myoinjury and muscle necrosis.
Results: In both models local inflammation increased depending on the number of injected Ly-$6C^{high}CD11b^+$ cells. Cell migration was analyzed by *in vivo* imaging, where fluorescent Ly-$6C^{high}CD11b^+$ cells were present only in inflamed tissue. Haematoxylin & Eosin analysis demonstrate a tissue improvement of animals treated with Ly6-$C^{high}CD11b^+$ monocytes.
Conclusions: In conclusion, our data demonstrate that we generate high amounts of Ly$6C^{high}CD11b^+$ monocytes *in vitro* with phenotypical plasticity, able to migrate to inflamed tissues *in vivo*.

P1615
Comparison of different protocols for the generation of monocytes-derived tolerogenic dendritic cells for immunotherapy

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Purpose/Objective: Therapeutic vaccination with tolerogenic dendritic cells (tolDC) has been shown to be a promising treatment in experimental models of autoimmune diseases. In this study we compared three different protocols for the generation of tolDC.
Materials and methods: We analyzed and compared the effects of PPAR-gamma agonist troglitazone (TGZ-DC), NF-kappaB inhibitor BAY11-7082 (BAY-DC) and a combination of dexamethasone and 1alpha,25-dihydroxyvitamin D3 (DexVD3-DC) during the generation of monocyte-derived DC regarding their phenotype, cytokine secretion and T cell stimulatory capacity.
Results: TGZ-DC and BAY-DC had a phenotype comparable to immature DC, while DexVD3-DC were more macrophage-like. Upon LPS stimulation, all DC populations had increased surface expression of MHC class II molecules and, except DexVD3-DC, also the co-stimulatory molecules CD86 and CD40. DexVD3-DC showed the highest expression of PD-L2 and co-stimulatory molecule CD80. Analysis of cytokine production using cell culture supernatants from all DC populations revealed that DexVD3-DC were the only cells not producing IL-12p70 upon LPS stimulation. IL-10 was produced by all unstimulated DC populations at a similar level. After LPS stimulation, DexVD3-DC produced the highest amounts of IL-10. To analyze the T cell stimulatory capacity of the different DC populations, the cells were co-cultured with autologous T cells using purified protein derivative (PPD) tuberculin as recall antigen. T cells co-cultured with DexVD3-DC proliferated the least.
Conclusions: DexVD3-DC have the best potential to be used in a tolerogenic dendritic cell-based immunotherapy for autoimmune diseases.

P1616

Dendritic cells in multiple sclerosis: key players in the immune pathogenesis, key players for new cellular immunotherapies

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Purpose/Objective: Much has been done to understand the pathogenesis of multiple sclerosis (MS). Whereas several studies have focused on the role of the adaptive immune system in the disease pathogenesis of MS, recent data suggest that dendritic cells (DC), which are innate immune cells, also contribute to the pathogenesis of MS. Due to their specialized antigen-presenting capacity, DC play an important role in polarizing the T cell response, thereby regulating the balance between immunity and tolerance.
Materials and methods: DC were generated *in vitro* from circulating monocytes of MS patients and healthy controls. Subsequently, phenotype, cytokine profile and T cell stimulatory capacity were investigated. Additionally, DC were exposed to pro-inflammatory signals to address their maturation-resistant phenotype.
Results: Our recent observations indicate that both circulating as well as *in vitro* generated DC of MS patients display an altered phenotype and function as compared to age- and gender-matched healthy controls, thereby influencing the effector function of T cells. Of interest, we also demonstrate that migratory patterns of DC in MS are altered, as evidenced by aberrant expression of migratory molecules, i.e. CD62L, CCR5 and CCR7, by DC of MS patients as compared to healthy controls. Furthermore, current immune-modulating therapies affect DC phenotype and function, hence underscoring their pivotal role in MS pathogenesis. In light of these observations, we subsequently aimed to modulate DC function in order to restore the immunological imbalance in MS. We demonstrated that treatment of DC with immunomodulatory cytokines (such as IL-10) results in a more stable tolerogenic DC phenotype. Indeed, modulation of DC by IL-10 treatment arrests DC in a semi-mature state and prevents the up-regulation of costimulatory molecules as well as the secretion of pro-inflammatory cytokines. Moreover, we deliver proof-of-principle that tolerogenic DC generated from MS patients are capable of suppressing myelin-specific T cell responses.
Conclusions: From the results of this study, it can be envisaged that we have provided the first step in the development of a new form of immunotherapy for the treatment of MS.

P1617

Functional modulation of human CD_4^+ T cells by polyclonal stimulation with a CD28 superagonist *in vitro*

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Purpose/Objective: Adoptive transfer of $CD4^+$ T cells plays a central role in many cell-based immunotherapies of which allogeneic bone marrow transplantations are probably the most successful. While the infusion of mature T cells offers the only chance of cure for many hematological malignancies it is also associated with Graft-versus-Host-Disease (GvHD) * a potentially lethal side effect. The pre-conditioning of the transplant recipients, however, also predisposes them to opportunistic infections like invasive aspergillosis. Therefore, novel therapeutic approaches for GvHD should prevent immunopathology while maintaining anti-infectious activity of the transferred T cells.
Materials and methods: Building on our own data generated in a pre-clinical mouse model of acute GvHD in which polyclonal stimulation of allogeneic T cells with a superagonistic anti-CD28 mAb *in vitro* prior to transplantation into allogeneic hosts *in vivo* protected from GvHD we now polyclonally stimulated human peripheral blood mononuclear cells (PBMC) either by anti-CD3 and anti-CD28 mAb-mediated co-stimulation or with a CD28-SA – both in the presence of recombinant human interleukin-2 (IL-2).
Results: While co-stimulation expanded $CD4^+$ and $CD8^+$ T cells, CD28-SA stimulation only drove proliferation of $CD4^+$ T cells. Both protocols, however, did not expand all $CD4^+$ T cell subsets equally but preferentially stimulated $CD45RA^-$ $CD25^-$ and $CD45RA^-$ $CD25^{low}$ memory cells as well as $CD45RA^+$ $CD25^{low}$ resting T_{reg} cells over $CD45RA^+$ $CD25^-$ naïve conventional T cells and $CD45RA^-$ $CD25^{high}$ effector T_{reg} cells. CD28-SA-expanded $CD4^+$ T cells differed from co-stimulated $CD4^+$ T cells as CD28-SA pre-stimulation was superior to co-stimulation in reducing allo-responsiveness of the $CD4^+$ T cells. Proliferation of memory $CD4^+$ T cells to recall antigens, including antigens of *Aspergillus fumigatus*, was, however, less affected by CD28-SA-mediated pre-activation. Cytokine skewing from IFNg towards IL-17 production further suggests induction of enhanced innate responses against *A. fumigatus* by CD28-SA-stimulated $CD4^+$ T cells.
Conclusions: Therefore, polyclonal stimulation of human $CD4^+$ T cells with a CD28-SA *in vitro* might reduce the risk of developing GvHD while maintaining the anti-infectious activity of the transferred T cells *in vivo*. This study was supported by BayImmuNet and by TheraMAB GmbH.

P1618

Generating human stem cell like central memory type EBV-specific $CD8^+$ T lymphocytes for effective and sustained cellular adoptive immunotherapy

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Purpose/Objective: Adoptive transfer of *in vitro* generated tumor- and virus-reactive T cells has evolved as a promising strategy in cellular immunotherapy to treat cancer and virus-associated malignancies such as Epstein-Barr Virus (EBV)-mediated post-transplantat lymphoproliferative disease observed after allogeneic hematopoietic stem cell transplantation. However, terminally differentiated, high avidity effector T cells (T_{EM}) exhibit limited homing and self-renewal capacity to establish sustained memory. In this study we thus explored the modulation of the canonical Wnt-signaling pathway previously shown to affect T cell differentiation to generate stem-cell like central memory-type (T_{SCM}), HLA-A*0201 restricted EBV-specific T cells.
Materials and methods: Naive $CD8^+$ T cells isolated from PBMC of HLA-A*0201 positive healthy donors using the *Naive CD8$^+$ T-Cell Isolation Kit* (Miltenyi Biotec) were repetitively stimulated with EBV-peptides (LMP2, BRLF1, BMLF1) presented first by autologous dendritic cells followed by $HLA\text{-}A2^+$ T2 cells or autologous EBV-transformed B cells (B-LCL) in the presence of the interleukins (IL)-2, -7, -15, -21 and the glycogen synthase kinase-3β (Gsk-3β) inhibitor TWS119 for 4 weeks. Phenotypic and functional analysis were performed by flow cytometry and IFN-γ ELISpot assay, respectively, at different time points of culture.
Results: Upon repetitive stimulation we obtained strong expansion of EBV-specific cytotoxic T lymphocytes (CTL) after 21–28 days of culture. These T cells expressed a recently described $CD8^+CD45RA^+CD45RO^-CD95^+CD27^+CD28^+CD62L^+$ $CCR7^+$ T_{SCM} phenotype when compared to CTL stimulated without inhibitor. First molecular studies

revealed elevated §-catenin levels suggesting a Tcf-7 mediated transcriptional effect on T cell differentiation. Interestingly, both TWS119 treated and control CTL populations elicited comparable reactivity against B-LCL *in vitro* as shown by IFN-γ secretion. Upon adoptive transfer in immunodeficient and IL-15 supplemented NSG mice EBV-reactive T_{SCM} showed prolonged engraftment. Further *in vivo* analyses on the cytolytic activity of the T_{SCM} EBV-CTL are in progress using NSG mice inoculated with B-LCL prior to adoptive T cell transfer.

Conclusions: In conclusion, this study demonstrates that T_{SCM} EBV-reactive CTLs with increased homing and self-renewal capacity can be generated from naïve T cells by modulating Wnt-signaling for improved immunotherapy.

P1621

Human adipose-derived stem cells exhibit a low susceptibility to NK cell-mediated lysis and inhibit their function

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Purpose/Objective: Mesenchymal stem cells (MSCs) have potent immunomodulatory capacities representing an interesting tool for cellular therapy. Although their interactions with Natural Killer (NK) cells have been previously studied in bone marrow-derived (BM)-MSCs, little information is available regarding adipose-derived MSCs (ASCs). To better understand hASCs-host interactions, we have studied the phenotype of hASCs and their capacity to regulate NK cell function.

Materials and methods: The hASCs were isolated from human lipoaspirates obtained after informed consent under the auspices of the appropriate Research and Ethics Committees. BM-MSCs were used as an MSC reference cell source. Phenotypic analysis of MSCs was done by flow cytometry. Purified NK cells were assayed for degranulation and IFN-γ assays or tested in co-culture experiments. Indoleamine 2,3-dioxygenase (IDO) activity was measured by determining both tryptophan and Kynurenine concentrations in supernatants from co-cultures of NK cells with hASCs.

Results: The hASCs displayed a lower susceptibility to NK cell-mediated lysis and a lower expression of ligands for DNAM-1 when compared with hBM-MSCs. NK cells produce IFN-γ in response to hASCs and hBM-MSCs. We demonstrated that hASCs and hBM-MSCs decrease NK cell degranulation against the susceptible target cells K562 through the action of soluble factors such as IDO.

Conclusions: Our *in vitro* results demonstrate that hASCs may display optimal characteristics for adoptive cell therapy in an allogeneic setting. We suggest that hASCs induction of NK cell tolerance can be mediated by soluble factors. Thus, the IFN-γ secreted by NK cells during NK/MSCs crosstalk may induce IDO expression that may exert effect in the immunosuppressive activity. These results suggest that for an adoptive cell therapy using allogeneic hASCs, the NK-hASCs crosstalk will not result in an immediate elimination of the transferred cells. Thus, hASCs may remain in the tissue long enough to balance the immune response before being cleared.

P1622

Human MSC support activated CD19+ B cell expansion: identifying cell contact dependent mechanisms

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Purpose/Objective: In order to develop, improve and license successful Mesenchymal stromal cell (MSC) therapy, it is essential to understand how transplanted MSC will interact with the host immune system. The immune-suppressive nature of MSC has been extensively characterised on human T cells and dendritic cells but the interaction between B cells and MSC remains controversial. This study characterised the effect of human bone marrow derived MSC on the activation and proliferation on allogeneic B cells isolated from healthy adult human donors.

Materials and methods: CD19+ B cells were isolated from PBMC using MACS beads. CD19+ B cells were activated with rhCD40L and CpG in the presence of pro-B cell cytokines IL-2, IL-21 and IL-10, for co-culture with human MSC. B cell activation was measured using flow cytometry (surface expression of activation markers) and real time PCR. B cell proliferation was determined using CFSE analysis.

Results: In the presence of MSC, the expression of CD69 and other markers by B cells was increased. MSC also significantly increased the expansion of CD19+ B cells, as illustrated by CFSE. MSC significantly increased the numbers of B cells entering first cell division, as well as the numbers undergoing extensive divisions (>5). This effect was dependent on direct MSC * B cell contact as shown using trans-well systems in co-culture assays. Preliminary data on candidate contact signals will be presented.

Conclusions: These results suggest that allogeneic human bone marrow derived MSC enhanced the activation and proliferation of CD19+ B cell populations *in vitro* through a contact dependent mechanism, and may limit the utility of MSC therapy for autoimmune or idiopathic conditions with a major B cell contribution to pathology such as SLE.

P1623

Induction of allo-specific IL-10-producing T cells by lentiviral vector-mediated IL-10 gene transfer

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Purpose/Objective: Type 1 regulatory T (Tr1) cells are a subset of $CD4^+$ regulatory T cells (Tregs) induced in the periphery characterized by high levels of IL-10 production and regulatory activity. During the last decade, Tregs cell-based therapy has become an attractive non-pharmacological therapeutic option for immunomodulation in different pathological settings. Although, several protocols to generate human Tr1 cells have been developed *in vitro*, the resulting population includes a significant fraction of contaminating non-Tr1 cells, representing a major drawback for clinical application of Tr1 cell therapy. To overcome this limitation we developed a lentiviral vector (LV) encoding for human IL-10 (LV-IL-10) and we demonstrated that enforced LV-IL-10-mediated expression confers Tr1 phenotype and function to human polyclonal $CD4^+$ T cells. In the present study we applied the LV-IL-10 platform for generating antigen-specific human Tr1 cells suitable for cell therapy.

Materials and methods: Human $CD4^+$ T cells were stimulated with allogeneic *in vitro* differentiated DC and activated T cells have been isolated based on the co-expression of early T cell activation markers. FACS-sorted T cells were expanded *in vitro* with IL-2 and transduced with LV co-encoding IL-10 and the marker gene, DNGFR. Resulting IL-10 engineered T cells were functionally characterized.

Results: The co-expression of early T cell activation markers allowed the selection of allo-specific T cells upon *in vitro* re-activation. FACS-sorted T cells can be easily transduced with LV-IL-10 and selected $DNGFR^+$ IL-10-transduced $CD4^+$ T cells expressed, upon allo-specific stimulation, high levels of IL-10, and are anergic. We are currently investigating whether IL-10 engineered T cells acquired suppressive activity.

Conclusions: We develop a suitable method to generate allo-specific IL-10 engineered T cells. These results represent the first step for the

development of antigen-specific IL-10-producing T cells and will contribute to increase the success of Tr1-based immunotherapy, inducing tolerance to selected antigens, while minimizing general immune suppression.

P1624

Lack of long pentraxin 3 (PTX3) in bone marrow-derived mesenchymal stem cells impairs wound healing in mouse

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Purpose/Objective: Although several studies have shown the capacity of mesenchymal stem cells (MSCs) to repair and regenerate different tissues, the mechanisms underlying these processes are not understood. In the present study we analyzed the role of the long pentraxin 3 (PTX3) in enhancing the wound closure. PTX3 is a multifunctional protein produced by MSCs after activation with inflammatory cytokines, which is involved in innate immunity, inflammation and extracellular matrix deposition.

Materials and methods: PTX3-deficient MSCs ($PTX3^{-/-}$MSCs) were collected from bone marrow of PTX3 knockout mice. After 3–5 culture passages, cells were tested for the expression of specific surface markers, their ability to differentiate into mesengenic lineages and their capacity to abrogate T cell proliferation. Finally, equal number of both $PTX3^{-/-}$ and WT MSCs were implanted into excisional wounds created by a biopsy punch on the back of allogenic WT and $PTX3^{-/-}$ mice. Wound area was measured up to 14 day and calculated using an image analysis program. The wound specimens were collected at 2, 7 and 14 days and processed for histology.

Results: By analyzing MSCs obtained from bone marrow of PTX3 knockout mice we demonstrated that, similarly to WT MSCs, $PTX3^{-/-}$MSCs displayed typical fibroblastoid morphology, were consistently devoid of contaminating hematopoietic cells and expressed common MSC markers. In addition, these cells were able to differentiate into adipocytes and osteoblasts, and drastically decreased the mitogen-induced proliferation of PBMCs, in a dose dependent manner. Importantly, in a mouse model of wound healing, $PTX3^{-/-}$MSCs showed an highly significant defect in wound closure compared to WT MSCs at each time point. Histologic evaluation of skin samples from mice treated with $PTX3^{-/-}$MSCs showed a reduction in the granulation tissue at day 7, a significant increase of polymorphonuclear cells in the wound bed and an enhancement of the fibrin deposition at the 2nd day after injury. Accordingly, $PTX3^{-/-}$MSCs also failed the ulcers closure in PTX3 knockout mice.

Conclusions: We demonstrated that PTX3 deficiency does not alter the phenotype or the capacity of MSCs to differentiate into mesengenic lineages; of relevance, the production of PTX3 represents an essential requirement for MSC ability of enhancing tissue repair.

P1625

Lentivirus-induced dendritic cells co-expressing GM-CSF, IFN-a and pp65 for boosting immunity against cytomegalovirus

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Purpose/Objective: Off the shelf vaccines such as proteins, peptides, and DNA for reducing the risks of cytomegalovirus (CMV) reactivation after hematopoietic stem cell transplantation have so far not shown major efficacy in clinical trials. One possible explanation is the underlying immune suppressed or immune dysfunctional status of the host, which might be partly alleviated with Donor Lymphocte Infusions (DLI). We are seeking to harness the effects of DLI, by providing the lymphocytes with improved homeostatic and antigenic stimulation provided by engineered induced dendritic cells (iDCs).

Materials and methods: We evaluated integrase-defective lentiviral vector (IDLV) co-expressing the differentiated cytokines GM-CSF/IFN-α in combination with CMV antigen, pp65. Overnight transduction of human monocytes with IDLV generated highly viable (>21 days) and immunophenotypically stable DCs, called Self-differentiated Myeloid derived Lentivirus-induced DC (SmyleDC).

Results: *In vitro* cultured SmyleDCs demonstrated stable secretion of GM-CSF/IFN-α and several endogenously produced inflammatory cytokines (IFN-γ, IL-2, -5, -6, -8). Expansion of multi-antigenic CMV-reactive effector and memory CTLs were obtained after a single *in vitro* re-stimulation with SmyleDCs co-expressing pp65 as evaluated by tetramer analyses and IFN-γ ELISPOT. In order to evaluate the effects of anti-CMV CTLs expansion *in vivo*, immune deficient NOD.$Rag1^{-/-}$.$IL2r\gamma^{-/-}$ (NRG) mice were immunized with SmyleDC/pp65 and, 7 days later, mice were infused with autologous $CD8^+$ T cells from a CMV seropositive donor. SmyleDCs enhanced T cell engraftment and rapid expansion of multi-antigenic pp65 reactive CTLs in peripheral blood and spleen.

Conclusions: Due to their capacity to precondition the host with a suitable homeostatic and antigenic environment prior to lymphocytes infusion, SmyleDC/pp65 is a promising immune regenerative cell product for the post-transplantation setting. Development of tricistronic lentiviral vectors and evaluation of potency and safety in humanized mouse models transplanted with human hematopoietic stem cells are ongoing for further clinical development.

P1627

Mesenchymal stromal cells isolated from bone marrow from multiple sclerosis patients reveals immunosuppressive properties

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Purpose/Objective: Introduction/objective: Mesenchymal stromal cells (MSCs) have shown immunosuppressive and immunoregulatory functions. These properties of MSCs suggest their potential role in tolerance induction in autoimmune diseases. We evaluated the anti-proliferative capacity of MSCs derived from multiple sclerosis (MS) patients, their ability to induce regulatory T cells and secrete IL-10 and TGF-β *in vitro*.

Materials and methods: MSCs were derived from bone marrow aspirates from MS patients ($N = 10$) and healthy controls ($N = 10$) and isolated by plastic adherence. For the inhibition assays, peripheral blood mononuclear cells (PBMCs) were labeled with CFSE and cocultivated (1:2, 1:5 and 1:10 ratios) with patients' and controls' MSCs MSCs in the presence of PHA at 37°C in a 5% CO_2 for 5 days. For the immune-regulation assays, non-labeled PBMCs were cocultivated with patients' and controls' MSCs. After 5 days of coculture, T-cell proliferation was assessed by CFSE method and the percentage of $CD4^+CD25^{hi}Foxp3^+$ was assessed by flow cytometry. IL-10 and TGF-β was measured in coculture supernatants by CBA flex kit. The results were analyzed by Mann-Whitney *t* test. This study was approved by the local ethics committee.

Results: We observed significant differences ($P = 0.002$) in proliferation mean percentage when comparing PBMCs plus PHA (71.3 ± 12.9%) with patients' MSCs plus PBMCs at 1:2 (33.3 ± 13.4%), 1:5 (39.1 ± 21.9%) and 1:10 ratios (46.6 ± 20.1%). There were no differences ($P > 0.05$) in percentages of $CD4^+CD25^{hi}Foxp3^+$ regulatory T cells recovered after 5 days of coculture with patientsÕ MSCs compared to controls. IL-10 and TGF-β secretion were significantly decreased ($P < 0.05$) in supernatants derived from cocultures with patients' MSC (IL-10: 53.8 ± 49.4 pg/ml; TGF-β: 59.4 ± 24.6 pg/ml) compared to controls (IL-10: 109.9 ± 64.6 pg/ml; TGF-β: 226.6 ± 153.2 pg/ml).

Conclusions: Although the MSCs isolated from MS patients were able to suppress T-cell proliferation, they showed lower secretion of IL-10 and TGF-β cytokines. These data suggest that MSCs from MS patients have immunosuppressive capacity *in vitro*, however more studies should be conducted before their use in autologous clinical applications.

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P1628

Modulation of helper T cell responses using recombinant FoxP3 transfection

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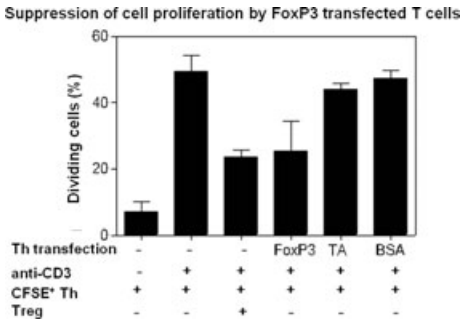
Purpose/Objective: Cationic polymer based transfection reagents are widely used as non-viral gene delivery vectors. The goal was to investigate if cationic polymers could be adapted for protein transport as well. Recombinant FoxP3 was chosen as a model protein for transfection into $CD4^+CD25^-$ T cells and potential induction of regulatory T cell phenotype and function.

Materials and methods: Full length FoxP3 gene was amplified by RT-PCR from cDNA of Balb/C mouse $CD4^+CD25^+$ cells. Protein was expressed in *E. coli* ER2566 strain and purified by affinity chromatography under denaturing conditions; activity was confirmed by EMSA. Mouse $CD4^+CD25^-$ T cells were transfected with recombinant FoxP3 using cationic polymer as transfection reagent. Protein localization after transfection was assessed by Western blot. T cell suppression was evaluated based on CFSE stained $CD4^+CD25^-$ T cell proliferation 72 h after activation with anti-CD3, ELISA assay was performed to monitor IL-2 levels in the culture medium.



Results: Recombinant FoxP3 was found in the nucleus 3 h after transfection. Transfected $CD4^+CD25^-$ T cells were able to suppress CFSE labeled $CD4^+CD25^-$ T cell proliferation to a similar extent as natural regulatory T cells (Fig). IL-2 secretion was blocked as well; however, this effect was highly dependent on FoxP3 transfection efficiency.

Conclusions: Cationic polymer can deliver functional FoxP3 protein and exert regulatory T cell phenotype to $CD4^+CD25^-$ T cells.

P1629

Optimization for contact time dependent effect of human mesenchymal stem cells on the CD_4 effector memory T cells

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Purpose/Objective: Mesenchymal stem cells (MSCs) have been known to have anti-inflammatory properties in various inflammatory diseases. However, the reported effects of MSCs on inflammation highly vary, which is partly attributed to the lack of standardized analysis. This study was aimed at analyzing contact time-dependent effects of human mesenchymal stem cells (hMSCs) on IL-17 and IFN-γ secretion by CD4+ effector memory T cells for the optimization.

Materials and methods: Bone marrow-derived hMSCs were co-cultured with human CD4+ T cells at a MSC:T cell ratio of 1:10. The suppressive effects of MSCs were evaluated by assessing their effects on the proliferation of CD4+ T cells by carboxyfluorescein diacetate succinimidyl ester (CFSE) stainingand the secretion of IFN-γ and IL-17A by CD 4+ memory T cells at different time points of contact and culture duration. The levels of IL-17 and IFN-g were determined in supernatants from the co-culture using commercial kits for enzyme-linked immunosorbent assay (ELISA).

Results: An earlier contact point between hMSCs and CD4+ T cells resulted in a greater suppressive effect on proliferation andboth IFN-γ and IL-17A secretionin an indoleamine 2,3-dioxygenase (IDO)-independent manner. While, a later contact point time of hMSCs to activated T cells after 3 days or longer and duration more than 4 days dropped most suppressive effect of hMSCs down on effector memory CD4 T cells.

Conclusions: Our study demonstrated that an earlier initial contact time, prior to the fully activated state of T cells, enhances the suppressive effects of hMSCs on effector memory CD4+ T cells. It also suggests that identifying an appropriate therapeutic window time would be critical in clinical trials with hMSC therapy for inflammatory disease.

P1630

Phenotypical/functional characterization of *in vitro*-expanded multipotent mesenchymal stromal cells from patients with type 1 diabetes

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Purpose/Objective: Mesenchymal stromal cells (MSCs) are progenitor cells with capacity to modulate the immune response, migrate to injury

site and promote tissue repair, thus representing a potential treatment for autoimmune/inflammatory diseases. However, it is not established whether MSCs have functional impairments in type 1 diabetes (T1D) setting. The objective of this study was to evaluate and compare phenotypical and functional characteristics from T1D-MSCs with control-MSCs for future clinical applications.

Materials and methods: MSCs cultures were derived from bone marrow aspirates of healthy controls (control-MSCs) and newly diagnosed T1D-patients (T1D-MSCs). T1D-MSCs were characterized using the following characteristics: morphology, circularity average (ViCell-XR® analyzer), expression of cell surface antigens (flow cytometry), adipogenic differentiation capacity (*in vitro* assay), gene expression profile by microarray analyses (Agilent Technologies) and migration capacity (*in vitro* assay). Results were compared with those of control-MSCs.

Results: MSC derived from T1D patients showed typical spindle-shaped morphology. The presence of surface markers (CD45, CD90, CD105, CD73, HLA-II) and cell circularity average (16.41 ± 0.69 μm T1D-MSCs × 15.91 ± 0.59 μm control-MSCs) did not differ from control-MSCs. T1D-MSCs exhibited the same differentiation potential towards the adipogenic lineage when compared to control-MSCs. Gene expression analyses reveled about 2978 differentially expressed genes ($P < 0.05$, fold change: 2). We observed an up-regulation of chemokine receptors genes (CCR3, CXCR5) and down-regulation of genes related with focal adhesion in T1D-MSCs. According to these results, MSCs from T1D-patients exhibit higher capacity to migrate through an 8μm membrane pore using 50% FBS as chemoattraent (786.1 ± 106.8 cells T1D-MSCs × 385.8 ± 61.02 cells control-MSCs, $P = 0.006$).

Conclusions: MSCs from T1D-patients exhibit the same morphological, immunophenotype properties and adipogenic differentiation potential as their healthy counterparts. On the other hand, T1D-MSCs exhibited differences in the gene expression profile and also a higher ability to migrate than control-MSCs. Further studies are needed to investigate the immunomodulatory/regenerative features of T1D-MSCs prior to their potential use in clinical trials.

P1631

Role of interferon-gamma in the activation of mesenchymal stem cells: analysis in a model of acute kidney injury

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Purpose/Objective: The stem cell therapy is flourishingas an alternative to acute kidney injury (AKI). It is known that in the lesion environment, pro-inflammatory cytokines, such as TNFα and IFNγ, activate the therapeutic role of MSCs. However little is known about this mechanism. This study assesses the role of IFNγ in the activation of MSC regenerative properties in acute renal models.

Materials and methods: MSC from IFNγ receptor knockout animals (IFNγR KO) and from wild type animals (WT C57/Bl6) were isolated from adipose tissue. After isolation and its culture, both cells were administrated in renal ischemia-reperfusion (IR) model. C57/Bl6 mice were subjected to bilateral IR by clamping both renal pedicles for 45 min. Four hour after reperfusion, 2.10^5 MSCs from IFNγR KO or from WT were intraperitoneally administered to each animal. After 24 h of IR, animals were sacrificed.

Results: Both treatments with MSC showed significant reduction of serum urea and creatinine, but the treatment with IFNγR KO MSC was less effective. Acute tubular necrosis (ATN) analyses corroborate with functional assays. Both cells treatments havereduced IL-6 mRNA expression when compared to untreated animals. However, IL-6 mRNA expression was greater in animals treated with IFNγR KO MSC when compared to treatment with WT MSCs. The IL-10 mRNA expression was higher in animals treated with WT MSC when compared to untreated and KO MSC group. PCNA analysis by immunohistochemistry was increased in treated animals with WT MSCs than animals treated with IFNγR KO MSC. At Tunel assays, it was observed that the group treated with IFNγR KO MSC had more apoptosis than others. In addition, *In vitro* studies have shown that IFNγR KO MSC have a lower proliferative rate than WT MSC. However, when WT MSC were incubated with recombinant IFNγ (20 ng/ml) the proliferative rate is increased.

Conclusions: The presence of IFNγ receptor at MSC is not essential for tissue repair, since functional parameters and ATN are not different in both cells treated animals. On the other hand, immunomodulation response differs. MSC response to IFNγ may play role on it, since proliferative rate and apoptosis are altered in the animals treated IFNγR KO MSC. More research is needed to better understand the role of IFNγ in activation and response of MSCs and their implication in cell therapy. Support: FAPESP, CNPq.

P1632

Salvage T cell therapy for resistant viral diseases after stem cell transplantation

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Purpose/Objective: Epstein-Barr virus (EBV), cytomegalovirus (CMV) and adenoviral reactivations are frequent complications after allogeneic SCT because of a lack of T cell control due to extensive immunosuppression. Cytotoxic T lymphocytes (CTLs) that recognize viral antigens are the most important immune effector mechanism controlling the persistent viral infections.

First-line treatment for viral reactivation is dose reduction of the immunosuppressive drugs and/or anti-viral therapy. For PTLD this is followed by rituximab (anti-CD20 monoclonal antibody). Despite these multiple treatment strategies, the l mortality from drug-resistant viremia after SCT is still considerable. Another treatment approach is adoptive transfer of virus specific CTLs from the donor. The standard method of adoptive T cell immunotherapy is laborious and time-consuming and is often too late to be administrated to the patient.

Materials and methods: We have developed a clinical separation protocol for virus specific CTLs based on labeling with multimeric complexes containing recombinant HLA molecules together with virus derived peptides. By combining this labeling technique with a secondary magnetic sorting we have managed to get a high purity of specific CTLs. This high purity diminish the risk of creating GVHD in the recipient even if the adoptive transfer of cells is done in an allogeneic or haplo-identical setting.

Results: We first used this protocol in an 18 year old patient with life-threatening PTLD. The patient developed an EBV associated lymphoma involving lungs, liver and both kidneys and also showed extremely high EBV titers in blood. It was decided to give her EBV specific CTLs from her mother. 2 months after the given EBV specific CTL infusion the EBV associated lymphoma was in complete regression. After this we have further successfully treated seven patients with life threatening viral disease (Adeno, CMV and EBV) with good efficacy. We could see a clinical and immunological response in six out of eigth patients. In five out of six of these responding patients we have been able to identify infused T cells using chimerism analysis and SNP markers specific for the CTL donor.

Conclusions: This method opens up the possibility to rapidly treat patients which are in acute need of T cell therapy and cannot wait for

prolonged expansion techniques or cannot tolerate standard treatment regiment.

P1634

T cells engineered to express high but not lower affinity T cell receptors recognize human tumor antigen cross-presented by the stroma and eradicate large tumors

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Purpose/Objective: Engineering of T cells with tumor-reactive T cell receptors (TCR) – TCR gene therapy – provides a powerful approach for adoptive therapy of cancer. The anti-tumor effect of TCR-engineered T cells is largely determined by the transgenic TCR that recognizes tumor cell-derived peptides presented on the patient's tumor tissue. However, current therapeutic approaches are impeded because although function of selected TCR can be analyzed *in vitro*, parameters that predict *in vivo* efficacy are not known. We developed a mouse model to evaluate the therapeutic effect of TCR gene therapy on established cancer.
Materials and methods: In order to establish a versatile, syngeneic model system for TCR gene therapy, we generated human MHC-transgenic mice that accept cancer cells modified to express human tumor antigens of choice. T cells were engineered with human tyrosinase-specific TCR of high or lower affinity. The anti-tumor effect of the T cells was analyzed *in vitro* and by adoptive transfer into mice bearing established, tyrosinase-positive cancer. Therapeutic efficacy was assessed by monitoring tumor growth. Cross-presentation of antigen by tumor stroma was analyzed in parallel by functional assays and by *in situ* confocal microscopy.
Results: Adoptive transfer of T cells engineered with high affinity tyrosinase-specific TCR resulted in complete rejection of cancer, whereas lower TCR affinity selected for escape variants and relapse. The tumor-derived human tyrosinase was cross-presented by tumor stroma cells. Although T cells engineered with both the high or lower affinity TCR showed identical tumor cell killing *in vitro*, only T cells expressing the high affinity TCR recognized cross-presented antigen on the tumor stroma.
Conclusions: This *in vivo* model provides a versatile, pre-clinical test system of adoptive T cell therapy of cancer to predict whether TCR-engineered T cells eradicate large tumors or select escape variants. Our results indicate that only TCR gene therapy with high affinity TCR prevents relapse, as opposed to use of lower affinity TCR which are typically isolated from the tolerant human repertoire against tumor-associated antigens. We suggest that the high affinity of the TCR enables the T cells to recognize and perhaps eliminate tumor stroma cells cross-presenting the melanoma antigen tyrosinase, resulting in bystander elimination of escape variants.

Poster Session: Gene Therapy

P1636

A good manufacturing practice procedure to generate therapeutic numbers of highly pure anti-leukemic virus-specific T-cells

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Purpose/Objective: Second half of 2012, we aim to start a clinical trial to treat patients with hihg risk acute leukemia with a donor-derived HA-1-TCR transduced (td) virus-specific T-cell product as early as 8 weeks after allogeneic stem cell transplantation. This T-cell product is selected based on well-defined specificities predicted to result in selective Graft versus Leukemia effect without Graft versus Host Disease.

Materials and methods: To obtain therapeutic cell numbers, one of the inclusion criteria is presence in donor peripheral blood of ≥1 virus-specific T-cell population with a frequency of ≥0.05% of T-cells. MHC-Streptamers will be used to isolate minimally one and maximally two virus-specific T-cell populations from donor leukocytes. MHC-Streptamer incubation will allow magnetic bead based cell selction of virus-specific T-cells of interest. Subsequently, these T-cells will be transduced with the HA-1-TCR. The process of isolation of pure populations of virus-specific T-cells and transduction with good manufacturing practice (GMP)-grade retroviral supernatant encoding the HA-1-TCR has been validated with three test procedures at large scale in the cleanroom.

Results: To pass the in process (IP) testing, T-cells needed to be ≥50% pure for the respective virus-specific tetramer after MHC-Streptamer isolation. In addition, release criteria for the cell product after transduction and subsequent culturing were positive staining for CD3/TCR$\alpha\beta$ ≥95% of the cells and ≥60% antigen-specific, as measured with virus- and HA-1-tetramers. Moreover, transduction efficiency of ≥5% as measured with HA-1-tetramers is a prerequisite.

Conclusions: All HA-1-TCR td virus-specific T-cell products met the criteria for IP testing and quality control testing, and were highly reactive against HA-1-positive leukemic cells. Here, we present a GMP-grade procedure, to generate therapeutically relevant numbers of defined antigen-specific T-cells.

P1638

Downregulation of CTLA4 by vector-mediated RNAi in human leukaemia T cells

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Purpose/Objective: In this study a gene therapy delivery system to overexpress small interfering RNA (siRNA) was designed and used to silence the expression of human CTLA4. We generated a stable T cell line expressing the CTLA4-specific siRNA that can be used as a tool for analysis of the role of CTLA4 in T cell down-regulatory pathways.

Materials and methods: A small hairpin RNA (shRNA) duplex designed to target the human CTLA4 mRNA transcript (siCTLA4) was cloned under the control of a U6 promoter in a previously described pBSU6neo vector. As a negative control a scrambled version of that sequence (scrCTLA4) was used. The efficacy of each plasmid was estimated by transient co-transfections in HeLa cells with a plasmid encoding CTLA4 expression, which was detected using Western blot and real-time PCR. Three stable T cell lines that have separately integrated pBSU6neo siCTLA4, pBSU6neo scrCTLA4 and pBSU6neo into the cellular genome have been developed and silencing efficacy was estimated by RT-PCR analysis. A stem loop RT-PCR method followed by real-time quantification was optimized to detect the expression of the mature siRNA in the new cell lines.

Results: We generated successfully a novel shRNA expressing vector pBSU6neo siCTLA4 encoding a sequence designed to silence CTLA4 expression. The ability of this vector to downregulate over 80% of induced CTLA4 expression compared to negative controls was confirmed in co-transfection experiments. Further stable T cell lines that incorporate the new plasmids were developed as a model to investigate the role of CTLA4 in functional assays. It was confirmed that the endogenous CTLA4 expression induced upon stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin is silenced in the T cell line which constitutively expresses the CTLA4-specific siRNA.

Conclusions: In this study we demonstrated the successful downregulation of human CTLA4 expression using RNA interference. We suggest that the new developed T cell line with silenced CTLA4 expression could be used as a new model for the investigation of the role of CTLA4 as a suppressor of T cell activation.

P1639

Enhancing the potency of plasmid DNA vaccine via the incorporation of CpG motifs

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Purpose/Objective: Plasmid DNA immunization is a potential approach against allergy, infectious diseases or tumors as it conferred many advantages over other vaccine approaches. In contrast to rodent models, most DNA vaccines exhibited low immunogenicity in non-human primates and humans. This study aimed at enhancing the immunogenicity of plasmid DNA vaccines by incorporating additional CpG motifs into the pVAX1 backbone.

Materials and methods: The D and K type CpG motifs were inserted into the pVAXBlot5 construct. The resulted plasmid, pVAXBlot5-DTKT, encoded a major allergen from the dust mite *Blomia tropicalis* for monitoring the antigen specific immune responses. The stimulatory properties of pVAXBlot5, pVAXBlot5-DTKT or DTKT oligonucleotides were evaluated by measuring the mediators released from the co-culture with human peripheral blood mononuclear cells (PBMCs). The prophylactic effects of the modified construct on Th2 immune responses were evaluated in a Blo t 5 induced murine allergy model. The immunogenicity of modified and unmodified constructs was also assessed in rhesus macaques by measuring Blo t 5 specific IgE and IgG as well as Blot 5 specific IFN-γ and IL-4 producing cells by ELISPOT assays.

Results: Co-culture of PBMCs with pVAXBlot5-DTKT elicited higher levels of pro-inflammatory cytokines/chemokines such as IL-6, TNF-α, MIP-1α and MIP-1β as compared to pVAXBlot5 or DTKT oligonucleotides stimulation at 5 and 24 h. *In vivo* evaluation in mice revealed that both pVAXBlot5-DTKT and pVAXBlot5 attenuated Blot 5 specific IgE. The modified pVAXBlot5-DTKT induced higher levels of Blot 5 specific IgG2c and IFN-γ when compared to pVAXBlot5. Monkeys immunized with both pVAXBlot5 and pVAXBlot5-DTKT and challenged with the Blot 5 protein showed lower Blot 5 specific IgE as compared to monkeys immunized with the pVAX1. The pVAXBlot5-DTKT immunized group also produced higher levels and fast kinetics of Blot 5 specific IgG when compared to those immunized with pVAXBlot5. Furthermore, the numbers of Blot 5 and Blot 5 peptides specific IFN-γ producing cells were higher from pVAXBlot5-DTKT immunized monkeys.

Conclusions: The optimal use of CpG motifs as the molecular adjuvant to enhance the priming of immune responses induced by DNA vaccine is still a feasible approach.

P1640

Entire requirement of endogenous type I interferon for the efficacious cancer treatment with viral vector-encoded interleukin-12

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Purpose/Objective: To date little is known about how the innate IFNα/β response affects the efficacy of cancer treatments based in viral vector delivery of therapeutic genes. Using Semliki Forest virus encoding IL-12 (SFV/IL-12), a promising RNA viral vector for tumor treatment, we have explored this.
Materials and methods: SFV/IL-12 was administered intratumorally to MC38 tumor-bearing mice and tumor growth, IFNα/β production and/or anti-tumoral CTL response were monitored. The IFNα/β production elicited by SFV/IL-12 was compared in WT, IPS1$^{-/-}$ and TRIF$^{-/-}$ mice. The role of macrophages, pDCs and conventional DCs (cDCs) in the IFNα/β response was analyzed by depleting these populations with clodronate or anti-mPDCA-1 mAb administration to WT mice or by treated CD11c/DTRGFP mice with Diphtheria toxin, respectively. Finally, the requirement of IFNα/β for the anti-tumoral efficacy of SFV/IL-12 was tested in mice deficient for the IFNα/β receptor (IFNAR) and in mice treated with a neutralizing IFNAR mAb.
Results: Intratumoral injection of SFV/IL-12 induces IFNα/β production which is impaired in IPS1$^{-/-}$ and, to some extent, in TRIF$^{-/-}$ mice. Using bone-marrow (BM) chimeric mice we show that both hematopoietic and stromal cells are involved in the IFNα/β response. Macrophages, pDCs and cDCs are all implicated in the IFNα/β production. The therapeutic activity of SFV/IL-12 against MC38 tumor is absolutely lost in IFNAR$^{-/-}$ mice and when IFNAR signaling is blocked *in vivo* with anti-IFNAR mAb. This is also true with other tumor model efficiently cured by SFV/IL-12, such as TC1. The lack of efficacy is not related to an impaired expression of the transgene because IFNAR$^{-/-}$ mice even express higher levels of IL-12 and other reporter SFV-encoding genes, such as Luciferase. Using BM chimeric mice were only hematopoietic or stromal cells were IFNAR$^{-/-}$, we show that the IFNAR absolute requirement is operational at hematopoietic-derived cells. In accordance with the described mechanism of action of SFV/IL-12, tumor-specific CTLs are outstandingly expanded upon SFV/IL-12 intratumoral injection. However, this does not happen when tumor-bearing mice are IFNAR$^{-/-}$, or have been treated with anti-IFNAR mAb, or if they are deficient for IPS1.
Conclusions: All in all, our data show that the efficacy of tumor immunotherapy with viral vector-encoded IL-12 is totally dependent on type I IFNs, unrevealing an unexpected mechanism of action of a therapy that is being translated to patients.

P1641

Exploiting CD8 co-receptor to improve the function of genetically modified CD4 T cells for cancer immunotherapy

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Purpose/Objective: TCR gene transfer can generate tumour antigen-specific T cells for adoptive immunotherapy. Many TCR genes isolated for tumour immunotherapy are MHC class-I restricted TCR and when transduced into CD8 T cells endow potent anti-tumour protection effects in-vivo. Attempts to engage the CD4 T cell using the MHC-I restricted TCR often resulted in suboptimal antigen specific responses. In this study, we show that the anti-tumour function of genetically modified CD4 T cells can be enhanced by introducing the CD8 co-receptor.
Materials and methods: The MHC-I restricted F5 TCR recognized influenza-A nucleoprotein (NP) presented by Db. Retroviral constructs containing the F5-TCR or the CD8 co-receptor were used to transduce murine CD4 T cells for in-vitro functional studies or in-vivo tumour protection studies. Irradiated mice were injected subcutaneously with tumour cells expressing NP and luciferase. These mice were subsequently injected with genetically modified CD4 T cells and the tumour growth was monitored. Surviving mice were re-challenged with irradiated tumour cells.
Results: The in-vitro function of CD4 T cells transduced with F5-TCR shows that IL-2 and IFN-γ production is enhanced with CD8 co-receptor. In addition, introducing a L58R mutation in the CD8 co-receptor can further increase this effect. In-vivo studies shows that introducing the TCR alone did not endow CD4 T cells with significant tumour protection. However adding CD8 co-receptor to the CD4 T cells enhances tumour protection. The genetically modified CD4 T cells persist for >3 months in surviving mice and when re-challenged with antigen the CD4 T cells with both F5-TCR and CD8 co-receptor have greater proliferative capacity and have more central memory phenotype cells.
Conclusions: The CD8 co-receptor enhances the in-vitro and in-vivo function of genetically modified CD4 T cells transduced with MHC-I restricted TCR. These genetically modified CD4 T cells can provide tumour protection, persist indefinitely and proliferate on re-challenge. The effects of the CD8 co-receptor can be further enhanced by introducing the L58R mutation.

P1642

Generation of human islet antigen-specific regulatory T cells for treatment of type 1 diabetes

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Purpose/Objective: Type 1 diabetes (T1D) is an autoimmune disease characterised by the destruction of insulin producing beta cells in the pancreas. Research has shown that the ability of natural regulatory T cells (nTregs − CD4+, CD25hi, FoxP3+) to suppress effector T cells in T1D is defective leading to the proposal of adoptive cell therapy, in which nTregs isolated from patients are enhanced for their regulatory capacity and re-infused, as a possible therapy. Support for this comes from the NOD mouse model of T1D where it has been shown that infusion of polyclonal nTregs can suppress disease with more effective suppression seen if the infused nTregs are specific for an antigen involved in disease. Therefore this research focuses on the development of methods for lentiviral transduction of T cell receptors (TCRs) into human nTregs, allowing for redirection of a patient's polyclonal pool towards antigens involved in disease.
Materials and methods: T cells with a known specificity were clonotyped in order to identify the allele usage of the V, (D) and J regions of the TCR chains and the sequence of the V region. To sequence TCR chains cDNA was produced from RNA via 5' RACE which was then used to produce TCR alpha and beta chains using constant region primers. Each cDNA product was then sequenced and re-amplified to add restriction sites for sub-cloning in to an expression vector for future production of lentivirus . This will involve the transfection of 293T cells with the viral backbone (product of sub-cloning), viral envelope and encapsidation plasmid using polyethylenimine.

Results: Clonotyping data has identified the sequences and allele usage of three TCRs specific for peptides of: the tyrosine phosphatase IA-2, insulin and haemagglutinin (HA). From these results V region primers were designed allowing for the alpha and beta chains of each of the TCRs to be re-amplified in order to add restriction sites to the cDNA products for subcloning in to the expression vector.
Conclusions: In conclusion, previous work has shown that addition of antigen specific nTregs can suppress the development of diabetes. Therefore the redirection of a patient's nTregs towards an antigen involved in disease may represent a powerful therapeutic tool. This ectopic expression of a TCR can be induced using lentiviral transduction and therefore the alpha and beta chains of three TCRs specific for antigens involved in T1D have been cloned to produce lentiviral vectors.

P1643

Immune responses to transgene and retroviral vector in patients treated with *ex vivo* engineered T-cells

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Purpose/Objective;Adoptive transfer of autologous T-cells that are gene-engineered to express antigen-specific receptors represents a promising experimental therapy to provide tumor-specific immunity to cancer patients. We studied safety and proof of concept of therapy with T-cells gene-engineered with a Chimeric Antigen Receptor (CAR) in patients with metastatic Renal Cell Carcinoma (RCC).
Materials and methods: In 12 patients with metastatic renal cell carcinoma (RCC), we performed a study with autologous T-cells genetically retargeted with a chimeric antibody receptor (CAR) directed towards carbonic anhydrase IX (CAIX), an antigen highly expressed in RCC. As we observed a limited (<30 days) peripheral persistence of the transferred gene-modified T cells we investigated the immunogenicity of these cells. Both humoral and cellular anti-CAIX-CAR immune responses were assessed followed by epitope mapping in case of detectable cellular responses.
Results: In the majority of patients we observed distinct humoral and/or cellular anti-CAIX-CAR T-cell immune responses. Humoral immune responses were anti-idiotypic in nature and neutralized CAIX-CAR-mediated T-cell function. Cellular anti-CAIX-CAR immune responses were directed to the complementarity-determining and framework regions of the CAR variable domains. In addition, two patients developed immunity directed against retroviral vector epitopes expressed by the therapeutic cells.
Conclusions: These observations may constitute a critical concern for clinical *ex vivo* g-retroviral gene transduction in general and CAR-retargeted T-cell therapy in particular, and underscore the need to attenuate the immunogenicity of both transgene and vector.

P1644

In vivo preclinical studies for the development of a gene therapy for IPEX patient

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Purpose/Objective: Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome is a lifethreatening disease due to mutations of the gene *Forkhead boxP3 (FOXP3). FOXP3* mutations cause the dysfunction of regulatory T cells (Tregs) that maintain tolerance to self-antigens. We previously showed efficient *in vitro* conversion of polyclonal T cells into polyclonal Tregs. Our goal is to validate, by *in vitro* and *in vivo* preclinical studies, the efficacy of these Tregs generated by lentiviral-vector- (LV)-mediated gene transfer of wild type FOXP3 in $CD4^+$ T cells ($CD4^{FOXP3}$) in view of a possible future therapy of IPEX syndrome.
Materials and methods: Conventional human $CD4^+$ T cells were transduced with a bidirectional LV, constitutively expressing FOXP3 under the control of the EF1a promoter and ΔNGFR as marker gene. Resulting $CD4^{FOXP3}$ were tested in a model of xenogeneic Graft-versus-host-disease (GvHD), induced by injection of $CD4^+$ T (Teff) cells into immunodeficient hosts: both NOD-SCID and NSG mice were used.
Results: Compared with NOD/SCID, NSG mice have been proven to be a more suitable model to test suppressive activity of $CD4^{FOXP3}$ cells, since they allow the induction of a xenoGvHD reaction with a lower number of Teff cells and gave more consistent results. At a ratio [Teff: $CD4^{FOXP3}$] of [1:1], $CD4^{FOXP3}$ cells efficiently blocked the development of xenoGvHD. $CD4^{FOXP3}$ have been proven to be efficient in arresting ongoing disease, even when injected in mice in which a xenoGvHD has been already established. Importantly, $CD4^{FOXP3}$ cells generated from IPEX patients were superimposable to $CD4^{FOXP3}$ cells derived from normal donors in controlling Xeno GvHD.
Conclusions: Our results showed that this protocol is efficient for the generation of $CD4^{FOXP3}$ cells suppressive *in vivo* both from normal donor and IPEX patients, and support the possibility of their future application for the therapy of IPEX and other autoimmune diseases.

P1645

Inducible TCR expression permits insights into functional properties of dual TCR T cells

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Purpose/Objective: T cell receptor (TCR) gene therapy is an option to treat cancer. However, due to the transfer of TCR genes into T cells, mixed TCR dimers composed of endogenous and transgenic (tg) TCR chains are formed. These mixed TCR possess unknown antigen specificity and severe transfer-induced graft-versus-host-disease was reported in a mouse model. We generated dual TCR T cells with regulable expression of one TCR and analyzed the mutual influence of both TCR with regard to cell surface expression, function, and pairing.
Materials and methods: We used LCMV- (P14) and ovalbumin-specific (OT-I) TCR and generated dual TCR T cells using mouse 58 T cells which do not harbor endogenous TCR. One TCR was constitutively expressed (mimicking the endogenous TCR). The expression of the second TCR was regulable (mimicking the tg TCR) using a tet-on retroviral vector. Depending on the expression level of the tg TCR we analyzed cell surface expression and functionality of both TCR and determined the occurrence of mixed TCR dimers by fluorescence resonance energy transfer (FRET). In addition, we analyzed the influence of TCR modifications (cysteineization (introduction of a second cysteine bridge), codon optimization) on TCR functionality and formation of mixed TCR dimers.
Results: In dual TCR T cells expression of the endogenous TCR was hardly decreased by expression of the tg TCR. However, functionality was strongly reduced or completely lost. FRET analysis revealed formation of correctly paired endogenous and tg TCR but also occurrence of considerable amounts of mixed TCR dimers which most

probably contribute to the loss of functionality. This result was independent of the expression level of the tg TCR. Cysteineization did not improve functionality or correct pairing of TCR in dual TCR T cells. However, in polyclonal T cells, optimized TCR formats showed an improvement in correct pairing and improved functionality in the presence of low amounts of cognate peptide.
Conclusions: Modifications of the tg TCR improved correct pairing of TCR chains. However, the level of mispairing depends very much on the selection of the endogenous and tg TCR. Increased levels of tg TCR did not avoid the formation of mispaired TCR. Thus, in TCR gene therapy, other strategies are needed to circumvent severe off-target side effects induced by mixed TCR dimers.

P1646
Matricellular protein CYR61 drives thymic stroma expansion and thymic output

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Purpose/Objective: T-cell development relies on functional microenvironments generated by thymic epithelial cells (TEC). These cells provide functional microenvironments to support the migration and proliferation of developing thymocytes and participate to their education. Decreased TEC numbers and impaired stromal architecture accompany age-associated thymic involution. Consequent decrease of T-cell production results in loss of repertoire diversity. Similarly, thymic structure and function are also severely damaged by infection, chemotherapy or radiation.

Therefore, modulating thymic stroma is of prime importance for the development of therapies to improve T-cell based immunity. In this context, we investigated whether matricellular protein CYR61 (CYsteine-Rich protein 61), previously implicated in cell proliferation, could improve thymus functions.
Materials and methods: Impact of CYR61 on TEC and T-cell production was evaluated *in vitro* and *in vivo* using thymocyte-depleted Fetal Thymic Organ Cultures (FTOC) and engraftment of CYR61 overexpressing thymic lobes CYR61 into athymic nude mice.
Results: Expression of CYR61 was strong in TEC and decreased over age. Secreted CYR61 constituted niches for specific subsets of thymocytes, enhancing their interaction with TEC. Treatment of thymic lobes with recombinant CYR61 expanded thymic stromal compartment by inducing thymocyte-independent TEC proliferation. Engraftment of CYR61-overexpressing thymic lobes into mice drastically increased thymic output by increasing the hosting capacity of expanded lobes for circulating progenitors.
Conclusions: CYR61 drives the development of thymic stroma, thus enhancing T-cell release to the periphery.

P1647
Multicistronic vector encoding optimized safety switch for adoptive therapy with TCR-modified T-cells

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Purpose/Objective: Adoptive immunotherapy with T-cells transferred with T-cell receptors (TCRs) with anti-tumor reactivity is a promising treatment for malignancies. TCR gene transfer poses different safety issues that might warrant the inclusion of a suicide switch. Improved affinity of TCRs resulted in unwanted on-target toxicity, and off-target reactivity directed against healthy tissue was observed due to mixed dimers. For our clinical study we want to use a high affinity TCR that displayed high reactivity against multiple different tumor cell lines. However, some low on-target reactivity was observed against mature dendritic cells and proximal tubular epithelial cells. Therefore, for clinical purposes this PRAME-TCR might warrant inclusion of a suicide gene as safety switch.
Materials and methods: Human CD20 has been proposed as a non-immunogenic suicide gene targeted by the widely used clinical-grade antibody rituximab and can additionally function as a selection marker. Previously, we have demonstrated that expression of CD20 and the TCR in two separated constructs resulted in profound expression of both molecules. However, transduction of two constructs leads to reduced transduction efficiency, and requires an extra selection step *in vitro* of the $CD20^+$ T-cells to avoid the infusion of T-cells that express the TCR but do not express CD20. To increase efficiency and assure solid co-expression of both the introduced TCR and CD20, the TCR α and β chains and CD20 gene sequences were linked with self-cleaving 2A sequences in a single multi-cistronic vector. However, we observed that transduction of cells with this multi-cistronic vector resulted in impaired expression of particularly CD20. To overcome this low co-expression, both the introduced TCR as well as CD20 were codon optimized.
Results: We demonstrate that transduction with a multi-cistronic vector encoding for both the codon optimized PRAME-TCR as well as codon optimized CD20 (PRAME-TCR_{CO}-$CD20_{CO}$) results in pronounced, concordant cell surface expression of both the TCR and CD20, whereas no antigen-reactive single TCR positive cells were detected. Our data demonstrate that rituximab efficiently eliminated the transduced T cells, resulting in the absence of PRAME-specific reactivity of PRAME-TCR_{CO}-$CD20_{CO}$ transduced T-cells after rituximab treatment.
Conclusions: Codon optimization of both the PRAME-TCR and CD20 was shown to result in profound co-expression of both molecules. This concordant expression allows the use of CD20 as a selective and efficient suicide switch.

P1649
Short-Interfering RNA evaluation in experimental mice rabies virus infection caused by variant 2 and variant 3

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Purpose/Objective: Rabies is an ancient disease and until now no effective treatment is available. Treatment using short-interfering RNA (siRNA) to inhibit rabies virus (RABV) replication showed promising results *in vitro*. Our purpose was to evaluate the efficacy of siRNA in treating mice experimentally infected with different street RABV strains.
Materials and methods: Three groups of 20 C57/BL6 mice, SPF, 4−6 weeks-old were inoculated in gastrocnemius muscle with three different RABV strains. A variant two isolated from a dog [dv2 (LD50 10−3.39/0.03 ml)], a variant two isolated from a human [hv2 (LD50 10−6.66/0.03 ml)] and a variant three isolated from a human [hv3 (LD50 10−6.66/0.03 ml)]. For each group, 10 mice remained untreated and 10 mice were treated with a mix of three different siRNA sequences (3.3 μM each) associated with lipofectamine (Brand'o *et al.* 2007) based on rabies virus N gene as a target. Animals received a single dose of siRNA mixture, via intraperitoneal route, 24 h post RABV inoculation (p.i) and were observed during 30 days. Cox Proportional Hazards models were used to estimate lethality rates and Hazard Ratios (HR) between groups.
Results: For dv2, lethality was 37.5% in the inoculated group and 50% in the siRNA group ($P = 0.71$; HR= 0.75); for hv2, lethality was 100% in the inoculated group and 70% in the treated group ($P = 0.27$;

HR = 0.57); For hv3, lethality was 60% in the inoculated group and 80% in the treated group ($P = 0.21$; HR = 1.97).

Conclusions: Efficacy of siRNA seems to be associated to the RABV strain once theresults of survival was variable in the groups submitted to siRNA and infected with different RABV strain. The siRNAs used were designed based on Pasteur virus N gene sequence, a fixed strain while in our study street RABV strains were used. Even considered as a conserved gene, studies showed significant genetic variability. A nearly perfect complementary sequence between siRNA molecule and the viral RNA target is necessary for mRNA cleavage. Our RABV N gene sequences showed 85.7−95.2% of homology between v3 and siRNAs sequences and 95.2−100% to v2, confirming this natural variability. In this study, a non-biological delivery system was used and an important point is the difficulty of siRNA delivery within CNS being this a major problem in practical therapeutic.

P1651

Turning mTOR down in TCR gene therapy: the path to long-term protection?

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Purpose/Objective: Gene transfer of TCRs recognising tumour antigens offer the possibility to produce large numbers of tumour-reactive T cells that can be used in immunotherapy. However, despite initial tumour killing, TCR-transduced T cells have impaired formation of a memory population resulting in tumour re-growth and lack of long-term protection.

Recently, the mTOR kinase has been implicated in memory differentiation of $CD8^+$ cytotoxic T cells. Antigen stimulation of naïve T cells activates mTOR that in turn orchestrates the change from oxidative to glycolytic metabolism and the activation of protein translation in order to cope with the proliferative burst necessary to generate an effector population. Nevertheless, attenuation of mTOR activity with low dose of rapamycin during viral infection increased memory cell differentiation. Memory fate seemed to rely on T cell-intrinsic mTOR activity, making it an appealing target for manipulation in TCR gene therapy.

In this study we aim to attenuate mTOR activity in TCR-transduced T cells to promote memory formation and long-term tumour protection.

Materials and methods: We use a TCR gene therapy model where mice are injected with EL4 lymphoma expressing the NP flu peptide and allowed to grow for varying days before adoptive transfer of T cells transduced with F5 TCR (recognising NP).

mTOR activity in T cells was reduced by either co-transduction with shRNAs against mTOR or Raptor or by *ex vivo* treatment with rapamycin (rapa).

Results: We observed that transient high dose rapa treatment during *ex vivo* T cell activation caused only a slight reduction on transduction efficiency. Rapa-treated F5 cells showed impaired interferon-gamma (IFNg) production when stimulated *in vitro* with EL4-NP and failed to protect *in vivo* from large 5-day old tumours. Paradoxically, rapa-treated F5 cells more efficiently rejected tumour in mice bearing 1-day old tumours.

Permanent mTOR inhibition using shRNA silencing against mTOR or Raptor severely limited the expansion of F5 cells in tumour-bearing mice.

Conclusions: These results suggest that mTOR inhibition during activation impairs the capacity of F5 T cells to eradicate large tumours due to reduced effector functions but this conditioning is advantageous when facing smaller tumours. On the other hand, permanent inhibition seems to be highly deleterious for T cell function.

Poster Session: Immune Suppression

P1653
Antigen-specific TCR-modified Treg for treatment of EAE

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Purpose/Objective: Adoptive transfer of regulatory T cells (Tregs) can be applied to treat autoimmune disorders, but several studies have demonstrated that treatment efficacy depends on their antigen-specificity as determined by the T cell receptor (TCR). However, the isolation of sufficient numbers of antigen-specific Tregs from the endogenous repertoire is technically difficult and most likely not feasible for most tissue-specific antigens. Therefore, we applied a novel method to overcome these difficulties and evaluated the capability of a gene therapy approach to generate high numbers of antigen-specific Tregs by TCR gene transfer into polyclonal Tregs.
Materials and methods: Using a retroviral vector, we transferred the genes for a TCR directed against myelin oligodendrocyte glycoprotein (MOG) peptide 35−55 into Tregs isolated from C57BL/6 mice and analyzed their capacity to inhibit MOG-induced experimental autoimmune encephalitis (EAE).
Results: Flow cytometry analysis revealed that the TCR was expressed in a high percentage of cells. Further, TCR-modified Tregs could suppress proliferation of effector cells *in vitro* in a MOG peptide-dependent fashion. Most importantly, adoptive transfer of very low numbers of TCR-modified Tregs * but not polyclonal Tregs * inhibited development of MOG-induced EAE in mice. By use of a marker gene we were able to detect the adoptively transferred Tregs in draining lymphoid organs and in the CNS and could show that their presence coincided with a reduction of MOG-specific IFN-γ- and IL-17-producing effector cells.
Conclusions: We have shown that TCR transfer is a suitable method to generate antigen-specific Tregs for therapy and that TCR-modified Tregs are superior to polyclonal Tregs in their capacity to prevent EAE.

P1655
Burn and/or sepsis-injury related immunosuppression is accompanied by disturbances in CD4+ T cell activation/responses.

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Purpose/Objective: Burn injury caused hypo-responsiveness of T cells has been shown via evaluations of proliferative and IL-2 production responses ex vivo, and their antigen specific stimulation *in vivo*. Down-regulation of expression and functions of MHC-II and costimulatory molecules on APCs (antigen presenting cells)/DCs (dendritic cells), CD80/86, and CD40, can adversely affect their interactions with complementary costimulatory molecules on T cells, CD28, CTLA-4, CD40L, and lead to impaired T cell priming/activation and responses. Such derangements in APC/DC and T cell interactions may contribute to burn-related hypo-responsiveness of CD4+ T cells. We hypothesized that immature DCs fail to adequately prime/activate naïve CD4+ T cell in the lymph nodes. Burn/sepsis injury leads to insufficient maturation of DCs during their migration from peripheral tissue sites to lymph nodes. Changes in DC functionality with injury probably occur in a DC subset that is responsible for activation and differentiation into Th1 type T cells through DC expression of IL-12. Insufficiently mature DCs exhibit down regulation of surface expression of MHC-II and CD80/CD86/CD40 molecules.
Materials and methods: Adult male rats were subjected to a 30% TBSA full-thickness skin burn (95°C, 8 s) (B), or skin exposure to 37°C (S); Sham (S) and burned rats (B) killed 74−78 h later, their mesenteric lymph nodes (MLNs) isolated and processed to purify DCs using a positive selection procedure with MACS microbeads. CD4+ T cells were isolated from MLN using IMMULAN beads.
Results: We found potential disturbances in interactions between T cells and APCs, specifically DCs costimulatory receptors/ligands contributing to CD4+ T cell deficits in burn and/or sepsis injured animals. The latter effects of DCs are suggestive of the effects of immature DCs with insufficient expressions of MHC II, and costimulatory molecules, CD80/86, CD40, and ICOSL. Burn injured rat MLN DCs had decreased surface expressions of MHC-II, CD40, and CD80/86. Moreover, burn-injured rat MLN DCs had impaired ability to prime naïve CD4+ T cells.
Conclusions: Overall, the results suggest burn and/or sepsis-injury causes disturbances in interactions between CD4+ T cells and DCs through primary and secondary costimulatory receptor/ligand molecules, contributing to CD4+ T cell deficits in burn and/or sepsis injured animals.

P1656
Characterization of the role of MALT1 in the development of multiple sclerosis

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Purpose/Objective: The paracaspase MALT1 is a cytosolic signaling molecule that plays a major role in lymphocyte activation and proliferation. MALT1 mediates antigen-induced signaling to the transcription factor NF-κB by functioning as a scaffold protein. NF-kB controls the expression of many target genes, which in turn mediate distinct events in the inflammatory response to host injury and which are frequently excessively activated in immune-mediated diseases and neoplasia. We previously found that MALT1 not only functions as a scaffold protein, but also holds proteolytic activity, which fine-tunes antigen receptor signaling. More specifically, MALT1 was shown to proteolytically inactivate the de-ubiquitinating enzymes A20 and CYLD, which function as a cellular 'brake' on immune signaling. We now further investigated the effect of MALT1 inhibition on the development of autoimmunity.
Materials and methods: The role of MALT1 was studied in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Immune responses and pathology were compared in MALT1 deficient mice and wild type mice. In addition, we investigated the effect of MALT1 protease inhibitors.
Results: Data obtained with MALT1 knockout mice as well as newly developed MALT1 specific protease inhibitors will be presented, illustrating a crucial role for MALT1 in T cell activation and in the early priming phase of EAE.
Conclusions: Our results illustrate the therapeutic promise of MALT1 inhibition in the treatment of multiple sclerosis and other immune-mediated diseases.

P1657
Chloroquine treatment reduces the severity of experimental autoimmune encephalomyelitis

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Purpose/Objective: We aimed to assess whether treatment with chloroquine (CQ), an antimalarial drug, is capable of reducing the clinical signs of EAE.

Materials and methods: Mice were treated with CQ (3 mg/kg) for 5 consecutive days prior to EAE induction. Clinical signs were analyzed daily for 30 days. Ten days after the antigen administration half of the animals were killed and Ag-specific cellular proliferation was analyzed in cultures of splenic leukocytes stimulated with MOG_{35-55}. At the end of the experiment, mice were killed and brains and sera were collect for analysis of inflammatory cells of the CNS and anti-MOG IgG, respectively.
Results: We observed a significant reduction in EAE clinical score and it was accompanied with a decrease in T cell proliferative response against MOG as well as a significant reduction of infiltration of inflammatory cells in the CNS and in serum anti-MOG antibody levels.
Conclusions: The data presented here corroborates with previous findings showing that chloroquine may have an immune-modulatory function, and we show for the first time that chloroquine treatment suppressed the clinical signs of EAE.

Ethical approval: All experiments were approved by the internal ethical committee (CEUA #2687-1).

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P1658

Danish innovation consortium: TREG – Center. Regulatory T — cells as a new treatment paradigm for immune disorders

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Purpose/Objective: International scientific groups and companies have shown an increasing interest in the use of Treg cells for clinical applications. The Treg-Center innovation consortium wish to establish the first technology platform worldwide, which offers the industry access to screening facilities which will allow them to develop novel therapeutics focused on the cure of immune disorders by re-establishing the immune balance. A major challenge for development and use of Treg cells for clinical applications is the lack of biological markers. The Treg-Center will additionally try to identify novel markers for Treg identification and isolation or for diagnostic and therapeutic applications.
Materials and methods: The consortium activities are structured around two work packages (WP). The aims of WPI is to develop screening platforms for Human Treg cell stimulating drugs exploring activity, induction, expansion and homing of Treg cells. Methods include: FACS sorting and phenotypic characterisation, BD fastimmune human regulatory T cell function assay, standard Treg inhibition assay (PKH26 proliferation); Homing assay (transwell system), Tr1 cell generation. The aims in WPII are identification and validation of novel markers in Treg cells. The types of markers explored will be proteins of membrane associated and intracellular origin as well as microRNAs. All activities are based on material from healthy donors or patients with Rheumatoid Arthritis.
Results: Selected data from WPI are presented illustrating nTreg sorting procedure, nTreg expansion and phenotypic characterization. The function of nTreg cells is illustrated by using the regulatory T cell function assay, BD fastimmune, which measures the expression of the activation markers CD154 and CD69 on effector T-cells.
Conclusions: The Treg-Center consortium wish to develop predictive screening platforms for *in vitro* analysis of Treg modulating drugs acting at various levels on Treg cells; (1) activity, (2) expansion, (3) de novo generation and (4) homing. Additionally, the Treg-Center wishes to identify novel Treg cell-specific markers of secreted, membrane-associated as well as intracellular origin with the purpose of identification, isolation and diagnosis of Treg cells.

P1659

Deficiency in ST2 receptor results in a more severe adriamycin-nephropathy

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Purpose/Objective: Adriamycin (ADM)-Nephropathy is an experimental model used to mimetic the clinical manifestations of human focal and segmental glomerulosclerosis (FSGS), a leading cause of chronic kidney disease. Different work associated FSGS with Th2 immune responses, suggesting a possible implication of the IL-33/ST2 axis in disease pathogenesis. The IL-33 is a member of the IL-1-like family of cytokines, described both as an alarmin and a differentiation factor for Th2 lymphocytes. Because the T1/ST2 receptor mediates IL-33 signaling, we decided to determine the influence of ST2 deficiency in AN pathogenesis.
Materials and methods: BALB/c WT and $ST2^{-/-}$ mice received, by a single tail vein injection with 10 mg (ADM)/Kg. The renal function was determined by the proteinuria/creatininuria ratio.
Results: Proteinuria and albuminuria are evident at day 4 post-ADM and progressed in a time-dependent manner. In the $ST2^{-/-}$ mice, the proteinuria ratio was twofold increased in comparison to wild-type group, indicating a more severe disease. The western blotting analysis of the renal tissue revealed a significant increase in the levels of phosphoAKT, JNK-1 and IkBalpha in $ST2^{-/-}$ mice in comparison to WT BALB/c, indicating a higher proinflammatory signaling.
Conclusions: Taken together our data show that the severity of AN increases in the absence of ST2 receptor, suggesting a regulatory role for IL-33 cytokine during FSGS pathogenesis.

P1660

Effect of allogeneic blood transfusion on peripheral blood Tregs and cytokine levels of patients that underwent joint replacement surgery

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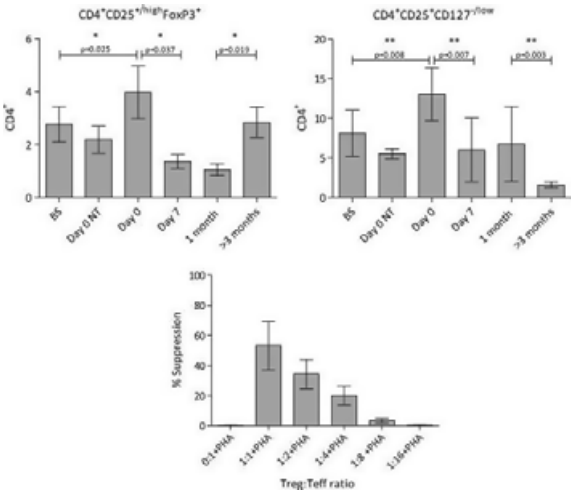
Purpose/Objective: Clinical and experimental studies have established that allogeneic blood transfusion (ABT) can cause immunosuppression. To identify immune parameters that contribute to this effect, we determined the effect of ABT on peripheral blood (PB) cytokine profiles and Treg numbers and function in a cohort of patients with no underlying pathologies.
Materials and methods: Heparinized PB samples were collected from 46 patients (7 M/39 F, 28–88 yo) that underwent joint replacement surgery. The samples were collected immediately before surgery (BS), and after surgery (AS) on days 0, 7, 1, and 3 months to 1 year. Thirty six patients received ABT and 10 did not. PBMC were isolated, and the numbers and % of $CD4^+CD25^+Foxp3^+$ Tregs and $CD4^+CD25^{high/+}CD127^{low/-}$ Tregs were determined by FACS. Tregs and T effectors (Teff) were isolated from patients on days 0–7 and Treg functional assays were performed by culturing Tregs with PHA-stimulated Teff at different ratios for 72 h with CFSE, and analyzed by FACS for proliferation. Cytokine levels were determined in plasma by a cytometric bead array assay for IL-2, IL-4, IL-5, IL-6, IL-10, TNF-α, IFN-γ, and ELISA for TNF-α, TNF-RI (p55/p60) and II (p75/p80), TGF-β1 and β2.
Results: Both, $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^{high/+}CD127^{low/-}$ Treg populations increased significantly on d0 AS and decreased on d7 AS to below BS levels, to achieve homeostasis >3 months in transfused patients. In contrast, Treg levels remained the same between BS and AS

in non-transfused patients. Functional assays showed that Tregs were functional post-ABT and could suppress Teff proliferation efficiently.

In culture, isolated Tregs secreted TGF-β1. All cytokines and TNF-RI and II plasma levels increased on d0 AS (IL-6, TNF-RI and II significantly), in transfused patients immediately AS, and decreased by d7 AS. In contrast, TGF-β1 levels decreased on d0 AS and increased by d7. No differences in cytokine or receptor levels were observed in non-transfused patients AS.



Conclusions: ABT induces the immediate production of plasma cytokines, especially pro-inflammatory IL-6 and TNF-RI and II that decreases by d7. In parallel, ABT induces the proliferation of Tregs. These Tregs are functional, and secrete TGF-β1 that reaches BS plasma levels by d7. The Tregs seem to be Th3 inducible Tregs. Homeostasis is reached by 3 months post-ABT.

P1661

Exosomal T cell suppressor factor inhibits the generation of reactive oxygen intermediates in murine peritoneal macrophages

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Purpose/Objective: Contact sensitivity (CS) reaction in mice is regulated by antigen specific T CD8$^+$ suppressor lymphocytes that produce biologically active exosomes carrying T cell suppressor factor (TsF). Macrophages (MF) as effector cells of classical CS reaction mediated by Th1 lymphocytes and as antigen presenting cells may be the target cells for TsF. The aim of our experiments was to define if exosomal TsF with different hapten specificity affect macrophages in the production of reactive oxygen intermediates (ROIs).
Materials and methods: Production of TsF (tolerogenesis) is induced by intravenous application of a high dose of hapten-labelled syngeneic erythrocytes 7 days before epicutaneous immunization with the same hapten (trinitrophenol or oxazolone). Peritoneal MF are induced by mineral oil i.p. injection in naïve or tolerized donors. The ability of MF to generate ROIs was measured as luminol-dependent chemiluminescence after *in vitro* activation of macrophages by zymosan (in case of naïve MF in the presence of hapten-specific T cell suppressor factors).
Results: MF from *in vivo* tolerized donors expressed the increased ROIs production in comparison to appropriate control. On the other hand the significant inhibition of generation of ROIs by MF was observed in the *in vitro* presence of either trinitrophenol- or oxazolone- specific TsF in comparison to untreated MF.
Conclusions: Experiments indicate the influence of TsF exosomes on MF dependent on the dose and mechanisms of exosomal stimulation. Low dose of TsF *in vivo* stimulation seems to activate natural cytotoxicity of MF which in turn may lead to elimination of CS T effector lymphocytes. The effect of *in vitro* higher dose stimulation with TsF suppresses MF activity. Present study was the first step to define the exact regulatory mechanisms induced by the interaction between TsFs and macrophages.

P1662

Generation of human antigen-specific regulatory T cells by MHC-class I restricted T cell receptor gene transfer

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Purpose/Objective: The adoptive transfer of regulatory T cells (Tregs) as a therapy in autoimmune diseases has been well demonstrated in mouse models of disease. In the case of Type 1 diabetes, this therapy could potentially prevent or dampen down the auto-reactive immune response to the islets of the pancreas, a process in which CD8+ T cells play a pivotal role. However, this therapy is limited by the number of antigen-specific Tregs that can be isolated and expanded from patients. To overcome this hurdle, lentiviral gene transfer technology can be utilised to engineer populations of Tregs to express any T cell receptor (TCR) of choice, re-directing their antigen specificity. Furthermore, by generating Tregs whose TCR is MHC class I restricted and islet antigen specific, these cells can be targeted directly to the site of the inflammation and similarly against the CD8$^+$ T cells that elicit a large proportion of islet cell damage.
Materials and methods: An HLA-A2 restricted CD8+ T cell clone specific for a peptide in the preproinsulin signal peptide region has previously been isolated from a type 1 diabetic patient and designated the 1E6 clone. In order to produce lentivirus, the packaging cell line HEK 293Ts are transfected with plasmids containing the 1E6 TCR, lentiviral packaging and integrating genes. Supernatant from these cells containing lentivirus is then used to transduce the TCRβ deficient Jurkat cell line JRT3 T-3.5.
Results: We have created a stably transduced Jurkat cell line expressing the 1E6 TCR, which led to the reassembly of the full CD3 complex on the cell surface. However MHC tetramers+ the 1E6 cognate peptide fail to stain these cells, suggesting that in the absence of the CD8 co-receptor, MHC and peptide cannot bind to cell surface of the 1E6+ cells. Furthermore, peptide specific activation of these cells shows that they cannot be activated by APCs expressing cognate peptide by both CD69 up-regulation and IL-2 secretion.
Conclusions: In conclusion we have demonstrated that we can generate stably transduced cells expressing a HLA-A2 restricted preproinsulin-specific TCR. However, our data has revealed a dependency on the presence of CD8 for this TCR to function. Thus, in order to transduce Tregs with an MHC class I restricted islet antigen specific TCR, a CD8 independent TCR will need to be identified to be used in this system.

P1664

Human dendritic cell based *in vitro* screening models mimicking Th1, Th2, Th17 and Treg responses

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Purpose/Objective: Dendritic cells (DCs) play a central role in regulating the immune system both under normal conditions, but also

under pathogenic conditions like autoimmunity, allergy and infection. This function of DCs makes them attractive target cells for therapeutic intervention in inflammatory diseases. We have designed human monocyte derived DC-based *in vitro* screening models for identification of anti-inflammatory compounds for use in Th1 or Th17 mediated autoimmunity or Th2 mediated allergic reactions. Further, we have setup a Treg platform, with tolerogenic DCs differentiating $CD4^+$ T cells into IL-10 secreting Tr1 cells, in order to evaluate Treg promoting compounds.

Materials and methods: Monocyte derived DCs were incubated with pro- or anti-inflammatory DC cocktails with or without prior addition of immune suppressive reagents. The DC cocktails were evaluated by FACS phenotypic analysis, ELISA cytokine measurement and T cell proliferation [mixed lymphocyte reaction (MLR)]. Generation of Treg cells was further evaluated by classical T cell suppression assay (PKH26 proliferation).

Results: Nine different Th1 cocktails consisting of TLR agonists, cytokines and lipids were designed based on their ability to induce secretion of IL12p70 and TNFa from DCs. All cocktails induced DC expression of HLA-DR, CD40, 80, 83 and 86, indicating development of the immature DCs into mature DCs. Furthermore, DCs were able to induce a Th1 response defined by high IFN-g secretion in a MLR. The model was validated with anti-inflammatory drugs at both DC and T cell level using dexamethasone (Dex) and rapamycin.

Two different Th17 cocktails were used to induce an inflammatory Th17 response. DCs stimulated with the Th17 cocktails secreted IL23 and differentiate CD4+ T cells towards an IL17A secreting Th17 phenotype in a MLR. Dex or the small molecule inhibitor STA5326 was used to validate the model.

A single Th2 cocktail was developed modulating DCs to promote a IL5 and IL13 secreting Th2 phenotype. The model was validated with Dex which was able to suppress the Th2 response.

Finally, a Treg platform was setup with tolerogenic DCs differentiating $CD4^+$ T cells into IL-10 secreting Tr1 cells.

Conclusions: We have designed human *in vitro* DC-based screening models for identification of immune-modulatory drugs for treatment of inflammatory or allergic diseases.

P1666
IL-2 and rapamycin alone or combined, friend or foe for type 1 diabetes?

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Purpose/Objective: Regulatory T cells (Treg) play a major role in controlling the pathogenic autoimmune process in type 1 diabetes (T1D). We have recently shown that a short course of low-dose IL-2 administration cures new onset T1D in non obese diabetic (NOD) mice, by specifically activating pancreas infiltrating Treg and reducing interferon-gammaproduction by pancreas infiltrating T cells. However, only 60% of mice afford remission and only half of them remain normoglycemic for lifelong. Here we attempted to improve the rate of T1D remission in NOD mice.

Materials and methods: For that we tested different high doses of IL-2 and the combination of low dose IL-2 and rapamycin (RAPA) in T1D.

Results: First, we tested the capacity of different IL-2 doses to prevent T1D. Surprisingly, only with a ten times increasing ofthe curative IL-2 dose, T1D development was accelerated and higher IL-2 dosis were toxic to the mice. Of note, NOD mice of different sex and ages showed a significantly different susceptibility to high-dose IL-2 diabetes acceleration or toxicity. Second, we tested the combination of IL-2 with (RAPA), which is an immunossupressor used in human transplantation and which can selectively eliminate effector T cells (Teff) while sustaining an increase of Treg *in vitro*. It has already been described that long-term RAPA treatment can prevent diabetes, so we directly tested the RAPA+ IL-2 combination in a curative schedulle in new onset diabetic NOD mice. Of note, RAPA+ IL-2 could not induce T1D remission, associated with an absence of control of the production of IFN-gamma by pancreas infiltrating T cells. Moreover, in IL-2- cured mice, RAPA annulated the T1D remission in a reversible way. We are now studying the underlying mechanism.

Conclusions: Our results have an impact on IL-2 and RAPA treatment on human T1D, as new clinical trials are ongoing or are been implemented using these drugs in patients.

P1667
Immunomodulatory effects of different hASC clonogenic populations on human PBMNc

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Purpose/Objective: Previous studies have shown that mesenchymal stem cells (MSC) are able to modulate the immune response of different leukocyte populations, i.e. Tregs, through mechanisms yet to be elucidated. Most of these studies have used heterogenic cell populations. However in the present study we analyze the immunomodulatory effect of five different MSC clones obtained from human lipoaspirates (hASCs).

Objectives: (1) To identify the membrane phenotype and Th1/Th2 cytokine production profile of each clone.

(2) To assess the modulating effect of clones on the proliferation of PBMNc in cocultures at different ratios (MSC:PBMC; 1:10 and 1:5)

(3) To establish differences between the behavior of the different clones, especially according to their specific immunomodulatory ability.

Materials and methods: Flow cytometry (FC: EPICS XL, Coulter, FACSCalibur, BD) anddirect immunofluorescence for membrane phenotype. Cytokines by FC, CBA techniques (BD) and Flow-cytomix (eBioscience). Proliferation through FC and CFSE technics (Sigma).

Results: All of the clones showed different intensities of autofluorescence (FITC emission spectrum) and different expression of CD90, CD105, CD73 and CD44 being negative for CD34 and CD45. All the clones secreted high to very high levels of IL-6 and IL-8 and mild to moderate levels of IL-1§ and TNF-α, both spontaneously and after LPS-stimulation. Two out of them also showed a slight secretion of IL-12 and INF-γ, and two more clones a slight secretion of IL-10 and/or IL-4. Cocultures carried out under different stimuli (LPS, PHA and anti-CD3/anti-CD28) showed different degrees of decrease in lymphocyte proliferation for all of the clones, reaching this immunosuppressant effect maximum values for one of these clones (two times more).

Conclusions: The Th1/Th2 profile as well as the membrane phenotype of each clone can influence in their ability to modulate various leukocyte populations. This different immunomodulating effects obtained by using clonogenic ASCs populations instead of heterogenic whole populations could be of interest for being applied with clinical purposes in the field of cell therapy.

P1668
Immunosuppression of ICR mouse strain with 5-FU administration

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Purpose/Objective: It has been already reported the experience with a variety of dosage schedules therefore an intravenous administration of

5-Fluorouracil (5-FU) is recommended, undiluted given by rapid injection of 15 mg/kg/day × 5 followed by 7.5 mg/kg on alternate days to the point of slight toxicity and so on. Our aim was to develop a half-lethal dose of 5-FU for ICR mouse strain, to determine the effect on the myeloid and lymphoid cells, to detect white blood cell abnormality.
Materials and methods: We've chosen virgin ICR female mouse strains, 2−2.5 months age. Each mouse had a 25 g weight. Intravenous administrations of 5-FU were in the following doses: 16, 32, 40, 48-mg/kg/day during four times in volume 100 μl for each time. Intravenous administrations of saline drip were used as a control. 5-FU was administered daily for 4 days, and every second day for 8 days. Blood sampling was performed before the introduction of 5-FU, and after the first administration on 5th, 7th, 12th and 30th days. Gorjaev's count chamber was used for measuring the number of white blood cells. For performing differential white blood cell counts May-Grunwald and Romanowsky stain and microscopy were applied.
Results: ICR mouse strain contains 10−15 million leukocyte cells per ml in a blood. After course of administration of 5-FU 48 mg/kg/day for during four times * the lethal dose was established. With the introduction of 40 mg/kg/day during 4 days, on the 5th day after the first injection of 5-FU, the total leukocyte count was from 3 to 4 millions cells/ml, which is three times less than normal. Also neutropenia was detected. The total number of leukocytes was restored to half rate, increased number of neutrophils detected. Doses of 32 and 16 mg/kg/day during four times and during every second day administration for 8 days didn't showed such significant results.
Conclusions: To determine the half lethal dose of 5- FU we used the following concentrations: 16, 32, 40, 48-mg/kg/day during four times in volume of 100 μl for each time. 40-mg/kg/day during four times reduced the total number of leukocytes to the minimum 3−4 million/ml. All animals were survived after this dose and restored their hematopoiesis in 30 days after first administration of 5- FU. Such kind of suppression may be used for further studies in the allogeneic transplantation and be helpful for engraftment of donor cells in the body of the recipient.

P1669
Investigating the role of splice variant CTLA-4 in cellular immunity

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Purpose/Objective: Cytotoxic T Lymphocyte Antigen 4 (CTLA-4, CD152) is a costimulatory molecule expressed on T helper cells which can bind to CD80 or CD86 molecules on antigen presenting cells and transmits an inhibitory signal to T cells. Various forms of CTLA-4 have been described in the literature. Our focus is mainly on a novel splice variant of CTLA-4 (svCTLA-4) produced by alternative mRNA splicing and importantly not by proteolytic digestion or shedding of membrane-bound CTLA-4. This transcript was first described by Magistrelli *et al.* in 1999 and it was demonstrated that unactivated human peripheral blood T lymphocytes could produce svCTLA-4. In contrast to membrane-bound CTLA-4, this protein lacks transmembrane and intracellular regions. Little is known about the immune regulatory functions of this protein.
Materials and methods: Aliquots of 2.5 × 10(5) PBMC were diluted in 1000 μl RPMI1640 supplemented with 2 mM L-glutamine, sodium pyruvate, HEPES, Penicillin & Streptomycin and 10% FCS. PBMC were incubated in medium alone or with PMA & ionomycin at a concentration of 10 ng/ml and 1 μg/ml at 37°C in a humidified atmosphere with 5% CO2. Cell supernatants were harvested every 24 h *in vitro* culture for further study of svCTLA-4 levels.

Ninety-six wells plates (Nunc Maxisorp, Thermo Fisher Scientific, Roskilde, Denmark) were used for svCTLA-4 ELISA. The coating antibody (Anti-human pan CTLA-4 antibody, BNI3 clone, BD Pharmingen) was added into each well & incubated at 37°C for 90 min. Plates were blocked with 3% BSA in PBS for 1 h at 37°C. Four replicates of samples were aliquoted into each well & incubated at 37°C overnight. A pool of high titre plasma was used as the standard & was diluted in 0.2% BSA-PBS.

After that, the detection antibody (Anti-soluble CTLA-4 biotinylated antibody, JMW-3B3 clone (specific for the splice variant form), obtained from Dr Frank Ward from University of Aberdeen) was added & incubated at RT in the dark for 2 h. Next, enzyme [Extravidin-alkaline phosphatase (Sigma-Aldrich)] was added and incubated for 90 min at RT in dark. Then, phosphatase substrate (Sigma Aldrich) was added into each well. Plates were read at 410 nm absorbance after 1 h of development.
Results: Our data demonstrates that this protein can be secreted by PBMC in *in vitro* culture. Upon stimulation of PBMC by PMA & ionomycin initially levels of svCTLA-4 were downregulated (until 48 h) in culture. SvCTLA-4 secretion was increased from 72 h in culture suggesting a possible role in controlling immune activation.
Conclusions: CTLA-4 is known to play an important role in mediating the function of regulatory T cells. Therefore, we would like to further investigate the effect of svCTLA-4 on T regulatory cell function.

P1670
Investigating the role of the aryl hydrocarbon receptor (AHR) in thymic-derived regulatory T cells (Tregs) *in vivo*

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Purpose/Objective: Recently, CD4+ Foxp3+ Tregs have emerged as key elements within the immune system, controlling the establishment and maintenance of immunologic tolerance and immune homeostasis. Many cells in the immune system express AHR and its activation has been shown to suppress a number of T cell responses *in vivo*. Interestingly, evidence suggests that activation of AHR induces Tregs that are suppressive in multiple *in vivo* models of autoimmune disease. Kynurenine (Kyn), a tryptophan catabolite constitutively produced by human tumor cells, was recently identified as an endogenous ligand of AHR. In this context, Kyn was shown to suppress the anti-tumor immune response and promote tumor survival. It was demonstrated that addition of exogenous Kyn to *in vitro* cultures led to an AHR-dependent increase in Treg induction. The purpose of this study was to determine if expression of AHR and interactions with endogenous ligands is important for generation, maintenance and function of thymic-derived Tregs *in vivo*.
Materials and methods: To test the importance of AHR expression on Tregs *in vivo*, bone marrow chimeras were produced using an equal mixture of AHR-deficient and Foxp3-DTR bone marrow to reconstitute lethally irradiated Foxp3-DTR recipients. Foxp3-DTR mice express the human diphtheria toxin receptor (DTR) under the control of the Foxp3 promoter. Injection of DT leads to ∼98% depletion of Foxp3-expressing cells. Upon depletion of AHR-sufficient Treg cells, all remaining Tregs would lack AHR while all other hematopoietic cell populations would include at least 50% wildtype cells.
Results: Congenic markers (CD90 and CD45) were used to identify the origin of each cell population and we found that both donors were represented in equal proportions in the CD4+ and CD8+ T cell populations. Both donors were equally present in the Foxp3+ CD4+ population of PBS treated mice suggesting that AHR-deficient Tregs had no competitive disadvantage in the steady state compared to wildtype Tregs. In addition, within 2 weeks following Treg depletion, the Foxp3+ CD4+ population returned to control levels and was nearly entirely comprised of cells originating from AHR-deficient donor. This

suggests that AHR-deficient Tregs were able to successfully respond to homeostatic pressure and fill the space left following Treg depletion.
Conclusions: Our results suggest that expression of AHR on Tregs is not required for the development and expansion of the Treg compartment *in vivo*. However, we have some evidence suggesting that expression of AHR might play a role in the function of these Tregs.

P1672

L-plastin phosphorylation: a novel target for the immunosuppressive drug dexamethasone in primary human T cells

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Purpose/Objective: Activation of naïve T cells requires costimulation via TCR/CD3 plus accessory receptors, which enables the dynamic rearrangement of the actin cytoskeleton and immune synapse maturation. Signaling events induced following costimulation may thus be valuable targets for therapeutic immunosuppression. Phosphorylation of the actin-bundling protein L-plastin represents such a costimulatory signal in primary human T cells. Phosphorylated L-plastin has a higher affinity toward F-actin. However, the importance of the L-plastin phosphorylation for actin cytoskeleton regulation upon antigen recognition remained unclear. Here, we aim to analyze whether phosphorylation of L-plastin is important for immune synapse formation and/or maturation and whether the immunosuppressive glucocorticoid dexamethasone plays a role in L-plastin phosphorylation and synapse formation.
Materials and methods: Human peripheral T cell isolation.
Primary T cell transfection.
Immune synapse via multispectral imaging flow cytometry.
Gel electrophoresis and Western blotting.
Cell cycle analysis and flow cytometry.
Cell viability assay.
Results: We demonstrate that phosphorylation of L-plastin is important for immune synapse maturation. Thus, expression of nonphosphorylatable L-plastin in untransformed human peripheral blood T cells leads to reduced accumulation of LFA-1 in the immune synapse and to a diminished F-actin increase upon T-cell activation. Interestingly, L-plastin phosphorylation is inhibited by the glucocorticoid dexamethasone. In line with this finding, dexamethasone treatment leads to a reduced F-actin content in stimulated T cells and prevents maturation of the immune synapse. This inhibitory effect of dexamethasone could be reverted by expression of a phospho-mimicking L-plastin mutant.
Conclusions: Our data introduce costimulation-induced L-plastin phosphorylation as an important event for immune synapse formation and its inhibition by dexamethasone as a novel mode of function of this immunosuppressive glucocorticoid.

P1673

Lipopolysaccharide-induced M2 to M1 macrophage transformation for IL-12p70 production is blocked by Candida albicans mediated up-regulation of EBI3 expression

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Purpose/Objective: The response of M1 and M2 macrophages to *C. albicans* in the presence of LPS stimulation has not previously been reported. In this study, we examined plasticity of macrophages transforming from M2 to M1 in the presence of LPS and the impact *C. albicans* has on this transformation.
Materials and methods: Bone marrow cells extracted from C57/black mice femur bones were cultured with either 10 ng/ml rmGM-CSF or 10 ng/ml of mlM-CSF/IL-4 for 7 days before seeding the cells into 24 well plates for LPS stimulation, with or without Heat-Kill *Candida* (HKC). Released cytokines were subsequently measured by ELISA and gene expression determined by real-time RT-PCR. In some experiments, cytokine proteins were examined by Western blot. To confirm intracellular EBi3/p40/35 protein interaction, IL-12p70 biscistronic expression vector and EBI3 expression vector were constructed in pcDNA3.1A. CHO cells were transfected with expression vectors before detection of the protein by ELISA, Western blot (WB) and/or Immuno-precipitation WB.
Results: M1 cells stimulated by *Candida albicans* produced much higher TNFα compared with M2 cells. LPS converted M2 macrophages to the M1 phenotype with higher IL-12p70 production. *Candida albicans* stimulated a higher Epstein-Barr Virus induced protein 3 (EBI3) which can couple with IL-12p35 to form IL-35, a heterodimer soluble protein (EBI3/p35) with an immune suppressing activity. M2 cells stimulated with *C. albicans* suppressed IL-12p70 production (induced by LPS) in a dose dependent manner. This suppression resulted from competition between EBI3 and IL-12p40 for IL-12p35 binding. EBI3 expression was higher in M2 compared with M1 cells. *Candida albicans* also induced higher levels of EBI3 expression than LPS in M2. However, LPS and *C. albicans* stimulated similar levels of IL-12p35 in M2 cells. This suggests EBI3 induced by *C. albicans* competes with IL-12p40 for p35, leading to suppression of LPS induced IL-12p70. To confirm this hypothesis, an IL-12p70 expression CHO cell line was cloned by transfection with a biscistronic IL-12p40 and p35. The over expressed EBI3 largely suppressed IL-12p70 secretion in all cell clones. This reduction of IL-12p70 production was associated with EBI3/p35 heterodimer detection in the cell culture medium.
Conclusions: This result demonstrated that *Candida* de-sensitises tissue M2 macrophages to transform to M1 phenotype in the presence of LPS, by suppressing IL-12p70 production. This may lead to the avoidance of an unnecessary Th1 response during the resolving phase of infection.

P1674

Low dose of cyclophosphamide alleviates the suppression of contact sensitivity response mediated by macrophages

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Purpose/Objective: Intravenous transfer of hapten-conjugated macrophages (Mf) to naïve recipients results in the suppression of contact sensitivity reaction (CS) as an effect of IL-10 secretion by donors' Mf and recipients regulatory T cells. A single administration of low dose of cyclophosphamide (CY) inhibits the suppression of CS response. The aim of our study was to test in the *in vivo* experiment how CY treatment influences both populations * donors macrophages and regulatory T cells in recipients and leads to the alleviation of suppression of CS reaction.
Materials and methods: To estimate CS oil-induced peritoneal Mf harvested from naïve or CY low dose (50 mg/kg) treated donors were labeled with TNP and transferred *i.v.* to naïve or CY treated recipients or recipients injected *i.v.* with anti-IL-10 and/or anti-TGF-b mAbs. Some grop7 days later ear thickness was measured just before the PCL challenge and 24 h later ear swelling responses were measured.
Results: Treatment with CY of Mf donors and/or recipients as well as recipients with anti-IL-10 mAb significantly inhibits the suppression while anti-TGF-β mAb therapy is inactive.
Conclusions: A single administration of low dose of CY into either donors or recipients restores the ability of Mf to induce significant CS reaction as a result of elimination of suppressive properties of Mf and/or depletion of population of regulatory T cells in recipients or

elimination of their suppressive activities. Observed inhibition was probably a consequence of decreased secretion of IL-10 by both populations of cells as a result of CY low dose treatment.

P1675

Macrophages present a regulatory phenotype after chronic cold stress in mice: correlation with increased 11ß-hydroxysteroid dehydrogenase expression

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Purpose/Objective: Susceptibility to infections, autoimmune disorders and tumor progression are strongly influenced by activity of endocrine and nervous system in response to a stressful stimulus. When this adaptive system is switched on and off efficiently, the body is able to recover from the stress imposed. However, when this system is activated repeatedly or sustained, as during chronic or excessive stress, an allostatic load is generated, which can lead to disease over long periods of time. Chronic stress has immunosuppressive effects as illustrated by increase in severity and duration of infectious diseases and by the impairment of immune response to vaccines. However, the cells and the mechanisms involved in this immunossupression are not completely known. We investigated the effects of chronic stress on main functions of macrophages.
Materials and methods: Balb/c mice were chronically stressed by 4°C/4 h during 7 days and macrophages were evaluated for their ability to phagocytosis, superoxide and cytokines production and stimulate T cells.
Results: We found that chronic cold stress in mice regulates macrophages functions to a regulatory pattern. This regulation is shown by diminished phagocytosis, lower TNF-α and IL-6 and higher IL-10 production. Besides, resting macrophages are able to stimulate T cells with a regulatory cytokines profile after chronic stress. Furthermore, macrophages of stressed mice have increased expression of 11β-hydroxysteroid dehydrogenase, an enzyme that converts inactive glucocorticoid into its active form, and have lower capacity to control *Trypanosoma cruzi* infection *in vivo*.
Conclusions: As stress is a common aspect of modern life and takes part in the etiology of many diseases, the results of this study are important to improve the knowledge about the neuro-immune-endocrine interactions occurring during stress and show the role of macrophages in immunosuppression induced by chronic stress.
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P1676

Mast cells are a target of tick saliva

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Purpose/Objective: Mast cells concentrated beneath the epithelial surface of the skin play an important role during feeding of blood-sucking arthropods including ticks. Since tick feeding period can exceed 10 days, active modulation of the host immune response by tick saliva is required for the tick to complete its blood meal. However, whether the tick saliva affects also mast cell physiology and functions was not known.
Materials and methods: We tested the effect of *Ixodes ricinus* tick saliva on the degranulation and cytokine production by ionomycin- and/or IgE receptor-activated bone marrow-derived murine mast cells.
Results: The most affected mast cell cytokine was interleukin 9; its production after 48 h upon saliva treatment was decreased by approx. 80% compared with the IL-9 production in control untreated cells. Interleukin-4 and IL-13 were also significantly inhibited by the saliva, whereas IL-6 levels were only moderately reduced. In contrast, production of TNF-α after 4-h co-culture with tick saliva was not affected. Saliva had also no direct impact on the level of mast cell degranulation.
Conclusions: We demonstrated the direct effect of tick saliva on the production of cytokines in mast cells; these effects can change the cytokine balance in the tick feeding site and contribute to the modulation of host immune responses.
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P1677

Notch family signalling between MSC and immune cells

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Purpose/Objective: Mesenchymal Stem or Stromal Cells (MSC) are immunomodulatory cells derived from bone marrow. MSC mediated immune modulation involves the induction of regulatory T cell populations and inhibition of dendritic cell maturation. This study sought to (1) determine the contact dependent signalling pathways between MSC and dendritic cells that block DC maturation; (2) identify the parallel signal between MSC and naïve CD4+ T cells leading to Treg induction; (3) determine the induction of tolerogenic DC by MSC.
Materials and methods: MSC and either DC or CD4+ T cells were used. An eGFP*FOXP3 reporter system was used to measure Treg induction. Notch signalling was blocked using Gamma Secretase Inhibitor (GSI) on either T cells or dendritic cells and expression of GFP-FoxP3 and maturation markers were examined by FACS. Immature DC co-cultured with MSC were pulled back after 48 h and co-cultured with CD4+ T cells from DO11.10 mice in the presence or absence of cognate antigen. After 72 h, proliferation was examined and Treg determined.
Results: Use of GSI prevented the MSC mediatedinduction/expansion of CD4+ FoxP3+ T cellsto controllevels. When GSI was used with DC and MSC, the immature DC characteristics sustained by MSC were lost. These DC induced regulatory T cells and inhibited antigen driven T cell proliferation in a Notch dependent manner.
Conclusions: These data demonstrate that the Notch-Notch ligand pathway is involved in MSC mediated induction of Treg. Inhibition of the Notch pathway abrogates MSC modulation of DC function but the exact receptors/ligand interaction here have yet to be determined. Using lentiviral transduction, a stable lines of silenced MSC were created. Using these cells the specific ligand involved in MSC immunomodulation can be determined.

P1678

Rapamycin inhibits innate and adaptive immune functions of human plasmacytoid dendritic cells

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Purpose/Objective: Rapamycin is an immunosuppressive drug used to prevent organ transplant rejection and cancer. Plasmacytoid

dendritric cells (PDC) are important in innate immunity as they are the principal producers of IFN-α, and in adaptive immunity by presentation of antigens to T cells. PDC are critically involved in immunity to viral infections and in the pathogenesis of auto-immune disorders like Systemic Lupus Erythematosus (SLE). Here we report that innate and adaptive immune functions of human PDC are differentially regulated by Toll-Like Receptors (TLR) and CD40, and that rapamycin inhibits both innate and adaptive immune functions of PDC.

Materials and methods: Human PDC were isolated from blood of healthy volunteers and stimulated with loxoribin, CpG or CD40L in the presence or absence of rapamycin and tested for their cytokine production and T-cell stimulatory capacity.

Results: Human PDC activated by TLR-7 or TLR-9 ligands produced high amounts of IFN-α, but exhibited a weak T cell stimulatory capacity. Conversely, PDC activated by CD40-ligation failed to produce IFN-α, but induced robust allogeneic T cell proliferation and effector functions, among which production of pro-inflammatory cytokines IFN-gamma, TNF-alpha and IL-17. A clinically relevant concentration of rapamycin (20 ng/ml) inhibited the phosphorylation of S6 and the production of IFN-α by PDC, and suppressed the capacity of PDC to stimulate allogeneic T cell effector functions. Reduction of T-cell stimulatory capacity was most pronounced when rapamycin was added during activation of PDC via CD40, and was associated with inhibition of CD40 expression on PDC.

Conclusions: Activation of human PDC via TLR stimulates their innate immune functions, while activation via CD40 stimulates their adaptive immune functions. Rapamycin inhibits both innate and adaptive immune functions of human PDC, and may therefore constitute a novel treatment option for SLE, but increase susceptibility to viral infections.

P1680
Soluble MHC class II antigens as potent regulators of the immune response

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Purpose/Objective: Soluble MHC-II (sMHCII) molecules are present in body fluids of healthy individuals and are considered to be involved in the maintenance of self tolerance, while they have also been related to various diseases. Their concentration has been shown to increase during *in vivo* antigen-specific tolerogenic stimulation. At the cellular level, sMHCII proteins compete with membrane MHCII for TCR binding and induce Src kinases activation. The aim of the present study was to define the physical and chemical properties of sMHCII and examine their ability to affect responsiveness and cytokine secretion profile of total spleen cells, total T-cells or CD4+ cells.

Materials and methods: Soluble MHC-II proteins were isolated from serum of mice tolerized with human serum albumin (HSA). Purity was verified through ELISA, SDS-PAGE, Western blot analysis and specific biosensor assays. Protein concentration was calculated using the Lowry method. Total spleen cells or T-cells were isolated from control or immunized BALB/c male mice, while CD4+ T-cells were purified using Flow Cytometry Sorter. The effect of sMHCII on the different cell populations tested as to cell proliferation and marker expression was evaluated using incorporation assays and Flow Cytometry analysis respectively. Cytokine production was tested by ELISA.

Results: The obtained results showed that the sMHCII proteins were capable to suppress an antigen-specific immune response by reducing the number of active cells. Thus, sMHCII induced control CD25+ and CTLA+ T-cell populations, while reducing CD28+ T-cells. Furthermore, it was shown that sMHCII induced secretion of the cytokine IL-10, while stimulating the proliferation of suppressive CTLA-4+ cells, through the negative regulation of CD4+ and CD28+ cells.

Conclusions: The results presented here showed that sMHCII can negatively regulate an antigen-specific immune response *in vivo* as well as *in vitro* by inducing immunosuppressive cell populations and mediators. It is proposed that sMHCII molecules could play a very important role in immune homeostasis, as they could mediate antigen-specific tolerance.

P1681
Structure and function of oxazolone specific T suppressor factor in contact sensitivity response

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Purpose/Objective: Contact hypersensitivity (CHS) in mice is mediated and regulated by different subpopulations of T-cells. High antigen dose induced-suppressor T (Ts) lymphocytes inhibit CHS reaction through release of soluble suppressor factor (TsF). The aim of our study was to establish the nature of OX-TsF, factor specific for CHS elicited by oxazolone (OX).

Materials and methods: OX-TsF production is induced in mice by i.v. immunization with OX, followed by skin sensitization with the same hapten. OX-TsF is obtained as a culture supernatant of Ts and may be purified by phenol-chloroform extraction into a mixture of nucleic acids (PCE) and then DNA (QDNA) and RNA (QRNA) fractions may be separated on chromatography columns. Alternatively, suppressive exosomes may be isolated from culture supernatant of Ts by ultracentrifugation. Biological activity of OX-TsF and its preparations was determined *in vivo* in CHS model.

Results: OX-TsF displays the ability to inhibit both the elicitation of CHS in naïve recipients of T effector cells (Tef) and ongoing allergy in actively sensitized mice. The function of OX-TsF was observed in both PCE and QRNA, but not QDNA, fractions of the tested factor and was characterized by a dose-response effect. RNA nature of OX-TsF was confirmed by its sensitivity to RNase A treatment. Additionally, electrophoresis on agarose gel proved that the RNA fraction of TsF comprises particles of the size of 10–90 nucleotides.

TsF performs its function in antigen-specific manner, which is observed in both supernatants and exosomes obtained from cultures of Ts induced by different haptens.

The mechanism of action of OX-TsF may tentatively involve apoptosis of the Tef.

Conclusions: It seems likely that the observed suppressive effect, mediated *in vivo* by short RNAs transported antigen-specifically in exosomes in the future may be used as a therapeutic approach to the diseases which result from the impaired function of effector T cells.

P1682
Study of the effect of CAMPATH on cord blood and peripheral blood cells

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Purpose/Objective: CAMPATH is an anti-CD52 monoclonal antibody used as an immunosuppressive drug that effectively depletes T cells in the pre-transplant conditioning regimen.

Materials and methods: CD52 expression of the resting and activated immune cells were checked. Both resting and activated samples were treated with CAMPATH and compared the the non-treated samples.

Results: CD52 expression on resting and activated peripheral blood (PB) and cord blood (CB) cells was studied. For resting CB cells, the

level of CD52 expression on B cells and T cells was very comparable (~569.2 ± 44.19), natural killer (NK) cells expressed the lowest level of CD52 (278.4 ± 31.64), and CD52 was not expressed on resting CB stem cells. For resting PB, B cells expressed the highest CD52 expression (540.8 ± 68.5), followed by T cells (433.1 ± 67.15), NKT cells (349 ± 74.67), and NK cells (301.4 ± 59.5). CD52 density was shown to be significantly higher in CB T cell subsets than PB T cell subsets except for Treg cells where CD52 expression was comparable in both CB and PB (~451.2 ± 53). In contrast, CD52 expression in activated cells was slightly higher in PB than CB, with T cell subsets expressing the highest level (~657.3 ± 27.70 and ~496.8 ± 4.872 respectively) followed by NK cells (~415.0 ± 16.15). However, both PB and CB activated Treg expressed similar level of CD52 density (~396.4 ± 9.948).

A viability study was designed to study the potency of CAMPATH in inducing PB and CB immune cell death. Although different concentrations were used, CAMPATH induced apoptosis and necrosis in a comparable manner, having the same effects on both CB/PB T and B cells. No difference was shown for both CB/PB NK cells. A maximum response towards CAMPATH treatment was observed approximately 24-h post administration for all cell types.

Conclusions: A better understanding of the impact of CAMPATH on immune cells would be useful to contribute to the future clinical application of the drug in order to achieve optimal efficacy.

P1683

T cell-selective mutated interleukin-2 AIC284 efficiently protects Lewis rats from experimental autoimmune encephalomyelitis

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Purpose/Objective: The cytokine interleukin-2 (IL-2) critically influences the survival and function of $Foxp3^+$ regulatory T cells (T_{reg} cells) *in vivo*. Therefore, targeting T_{reg} cells via the high affinity IL-2 receptor, CD25, constitutes a novel therapeutic approach for autoimmunity. As anti-cancer therapy with IL-2 has revealed substantial toxicities due to activation of conventional memory T cells and NK cells expressing intermediate affinity IL-2 receptors, a mutated human IL-2 molecule, termed AIC284, has been developed to reduce these side effects.

Materials and methods: AIC284 shows similar binding to the human high affinity IL-2 receptor as wild-type (wt) IL-2 but about 10^4-fold less binding capacity to the intermediate affinity IL-2 receptor. In search for an appropriate rodent animal model to analyze the therapeutic potential of AIC284 in autoimmunity we observed that AIC284 only bound less well (100-fold) than wt IL-2 to intermediate affinity IL-2 receptors of the rat but not to those of the mouse. Therefore, we treated rats immunized with guinea pig myelin basic protein (gpMBP) to develop experimental autoimmune encephalomyelitis (EAE) for the first 4 days after the immunization with daily doses of 100 mg AIC284 or wt human IL-2 or 250 ml phosphate-buffered saline s.c.

Results: Treatment with AIC284 or wt IL-2 protected the rats similarly well from EAE, both, clinically and histologically. Further analyses revealed that application of AIC284 and wt IL-2 inhibited the generation of gpMBP-specific pathogenic, i.e. IFNg-producing, $CD4^+$ T cells and strongly reduced the infiltration of leukocytes into the spinal cord (SC). As expected, protection from EAE was associated with increased frequencies and absolute numbers of $Foxp3^+$ T_{reg} cells among $CD4^+$ T cells.

Conclusions: As far as the precise therapeutic mechanism is concerned, wt IL-2 and AIC284 appear to differ as wt IL-2 strongly expanded T_{reg} cells expressing the activation marker CD8 within secondary lymphoid organs (SLO) while AIC284 induced only a slight increase in $CD8^+$ T_{reg} cell numbers in SLO but instead favored their accumulation in the SC. Experiments directly addressing the contribution of CD8-expressing T_{reg} cells in AIC284-mediated protection from EAE are ongoing. This study was supported by a grant from AiCuris GmbH & Co. KG, Germany.

P1684

The compartment of human natural $CD4^+CD25^{++}CD127^-$ regulatory T cells is filled with cells reactive to various antigens

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Purpose/Objective: The population of regulatory $CD4^+CD25^{++}CD127^-FOXP3^+$ T cells (Tregs) is composed of thymus-derived (natural, nTregs) or Tregs induced in the periphery (iTregs). nTregs have a highly demethylated TSDR *FOXP3* and are believed to be important for control of autoreactive T cells. Tregs are important to control alloreactive T cells and could be used for cell therapy to induce tolerance to an allograft. Recognition of natural alloantigen-specific Tregs could facilitate a targeted approach for such a therapy. Expression of CD40L (CD154) can be used to detect antigen-specific $CD4^+$ T cells. In this study, we used specific expression of CD154 to study the presence of antigen-specific Tregs.

Materials and methods: Highly enriched fractions of Tregs and $CD4^+$ effector T cells ($CD4^+$Teff) were sorted and stimulated with various antigens in the presence of aCD40 to maintain CD154 on the cell surface. Subsequently, these CD154-expressing Tregs were sorted and tested either immediately for their suppressive capacity or following polyclonal (using CD3/CD28 beads) or antigen-specific expansion, respectively. The demethylation of TSDR *FOXP3* was determined for the different Treg-fractions. In addition, in depth phenotypic analyses were performed on the different Treg-fractions.

Results: Using different viral, vaccination as well as alloantigens, we detected similar frequencies of antigen-specific $CD154^+$T cells in both fractions with great specificity and sensitivity. Ag-specific $CD154^+$Tregs had a memory phenotype, no cytokine production after polyclonal stimulation, stable FOXP3 expression and a highly demethylated TSDR *FOXP3*. Sorted $CD154^+$ Tregs were superior in suppressing Ag-specific responses when compared to $CD154^-$Tregs. $CD154^+$ Tregs could be efficiently expanded in an Ag-specific manner, which enhanced their suppressor activity. After booster vaccination, the ratio of Ag-specific $CD4^+$ Teff:Treg temporarily increased substantially due to an increase in $CD154^+CD4^+$ Teff but not Tregs.

Conclusions: These data show for the first time that the compartment of circulating human nTregs is filled with Ag-specific T cells for a variety of antigens. Isolation and expansion of Ag-specific nTregs may be of potential benefit for Treg-therapy to induce tolerance in the setting of kidney transplantation.

P1685

The effect of all-trans retinoic acid on histocompatibility receptor distribution at the foeto-maternal interface

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Purpose/Objective: The field of reproductive immunology was created for understanding the tolerance strategies by which the semiallogenic foetus evades the maternal immune system. Multiple mechanisms underlie the tolerance shown by the maternal immune system. A chief role is played by the Human Leukocyte Antigen -G (HLA-G), a member of the non-classical Major Histocompatibility

Complex (MHC) class I molecule. HLA G has a unique pattern of expression; low polymorphism and tissue distribution that enable it to aid the process of fetal implantation by preventing it from being recognized as 'foreign'. In early work[1] it was shown that the MHC receptors (HLA-G and HLA-E) and that their distribution is linked to the cytoskeleton. Certain retro-retinoids are known to induce growth arrest and death by apoptosis in cell lines. It was demonstrated[2] that F-actin is a functional target for Retro-Retinoid Action. Hence, F- actin reorganization is an early event in RA induced apoptosis. Lack of MHC class I a and the expression of these non classical antigens has long been hypothesized to be a major factor in immune tolerance. This study is mainly focused on investigating the expression of HLA class I b, and assessing whether Retinoic Acid plays a role in the regulation of the functional expression of HLA-G.

Materials and methods: The techniques used in order to establish this study are flow cytometry, protein analysis and microscopic analysis.

Results: Our results revealed that Retinoic Acid up regulates surface HLA-G expression significantly in a dose and time dependant manner. Proliferation and Cytotoxicity assays further confirmed this effect and results were supported by the fact that the effects of ATRA could be inhibited by various concentrations of 9-cis RA, the isomer and antagonist of ATRA. Image analysis with Nikon Viewer software identified changes in morphology in HLA-G expression after ATRA induction, includingminor changes in the cytoskeleton.

Conclusions: All trans Retinoic Acid acts as a stimulator at certain concentration for HLA-G, and can induce apoptosis beyond 50 μM. The regulation of the functional expression of HLA-G by Retinoic Acid could induce a physiological role such as apoptosis. This study is also, a novel approach for studying ATRA mediated (using cytoskeleton as a functional target) apoptosis in embryonic cell lines. As the results were consistent in both cell lines, we can also propose ACH-3P as a model cell line for trophoblast investigation.

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P1686
The influence of opioids treatment on the immune response in mice: the role of macrophages

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Purpose/Objective: Immune responses mediated by lymphocytes and macrophages could be modulated by opioids (OPs) via their cell surface receptors. The aim of our study was to establish the role of opioid (morphine, fentanyl or methadone) treated murine macrophages (MF) in the regulation of humoral and cell-mediated responses in CBA/J mice.

Materials and methods: For this purpose macrophages obtained from opioid treated mice were either fed with sheep erythrocytes (SRBC) or labeled with TNP hapten and transferred i.v. into naïve recipients. Humoral response was estimated by plaque forming assay (PFA) and direct haemagglutination assay (HA) determining sera titres of anti-SRBC IgM and IgG antibodies. Contact hypersensitivity reaction (CHS) was tested by ear swelling measurement after challenge withTNP/PCL hapten. Macrophage properties *in vitro* were assessed in FACS analysis of surface markers expression, ELISA for cytokine secretion and chemiluminescence assay for oxygen intermediates (ROIs) production.

Results: The induction of humoral response by opioid treated Mf was strongly inhibited. Numbers of PFC were lower by 30% (morphine or fentanyl treatment) to 57% (methadone) as compared with control animals. This same holds true for Ab titers. CHS reaction was differentially affected by OPs. While all tested OPs increased the early phase of reaction, the late phase is increased only in morphine therapy and affected by fentanyl and methadone. The impairment of MF antigen-presentation ability was also observed as decreased expression of CD80, CD86 and MHC class II in FACS analysis. The secretion of proinflammatory cytokines by MF obtained from donors treated with OPs was inhibited andmorphine or methadone but not fentanyl therapy caused an enhancement of IL-10 as well as ROIs production by MF.

Conclusions: MF are the target cells for opioid activity in modulation of immunity. The inhibitory action of OPs on humoral and cellular responses seems to be an effect of impaired ability of MF to present antigen.

P1687
The role of Dickkopf-3 in immune responses

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Purpose/Objective: It is so far unknown to which extend tissue cells influence the type and strength of a local immune response. Recently, we demonstrated that Dickkopf-3 (Dkk3) is essential to maintain peripheral CD8 T cell tolerance in a TCR-transgenic mouse model (Papatriantafyllou, M; Proc Natl Acad Sci U S A, 2012, 109:1631–1636). Since Dkk3 is highly expressed by tissue cells of immune-privileged sites, as e.g. the brain and the eye, it is of interest whether the local production of Dkk3 contributes to immuno-suppression.

Materials and methods: *In vitro* T and B cells were stimulated and proliferation and secreted cytokines were analyzed. For these experiments either Dkk3$^{-/-}$ versus wt cells or addition of exogenous Dkk3 versus control were compared.

In vivo EAE was induced in Dkk3$^{-/-}$ and wt mice. T cells were purified from the brain at the peak of disease or during the recovery phase and analyzed. To investigate B cell responses Dkk3$^{-/-}$ and wt mice were immunized and antibody titers were determined at different time points.

Results: In order to investigate whether Dkk3 has an impact on immune responses in the brain, experimental autoimmune encephalitis (EAE) was compared in Dkk3$^{-/-}$ and wt mice. Animals lacking Dkk3 exhibited a prolonged disease compared to Dkk3 sufficient animals. This was associated with changes in T cell subset composition and the local cytokine milieu.

Comparing immune responses in organs lacking Dkk3 expression as the spleen by *in vivo* cytotoxicity assay no general state of hyperresponsiveness in Dkk3 deficient T cells was observed. *In vitro* we found a decrease in proliferation, expression of CD25 and secretion of IL-2 upon stimulation of T cells in the presence of exogenous Dkk3.

B cell responses were also affected by Dkk3. Mice deficient for Dkk3 showed altered antibody responses after immunization with TNP-BSA or TNP-Ficoll in comparison to wt mice. Furthermore, B cells from Dkk3$^{-/-}$ mice showed changes in cytokine expression *in vitro*.

Conclusions: Based on these data we conclude that Dkk3 is modulating T cell responses and antibody production.

P1688

Tolerization of diabetogenic CD4+ T cells by intranasal treatment with CTA1R7K-DD containing specific peptide through the induction of FoxP3$^+$ Treg cells

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Purpose/Objective: Type I diabetes (T1D) results from immune destruction of insulin producing β-cells in the pancreas islets. Diabetogenic CD4$^+$ T cells are key cells in the autoimmune process. To achieve tolerization of diabetogenic CD4$^+$ T cells would therefore be an important advancement in the development of treatment of T1D. We previously described that a mutated (R7K), enzyme killed, form of the cholera toxin A1 subunit based adjuvant CTA1R7K-DD induces specific tolerance rather than enhancement of immunity. Intranasal (i n) treatment with CTA1R7K-DD containing a type II collagen peptide reduced *in vitro* recall responses to the peptide, and moreover, ameliorated collagen induced arthritis in mice. Here, we use CTA1R7K-DD to investigate tolerization of diabetogenic CD4$^+$ T cells of the non-obese diabetic (NOD) mouse, exploring diabetogenic TCR transgenic BDC2.5 CD4$^+$ T cells.
Materials and methods: NOD or TCR transgenic BDC2.5 NOD mice were treated i n with CTA1R7K-*peptide*-DD fusion proteins, where the inserted peptide was Ins9-23 or PS3 (a strong agonist peptide for the BDC2.5 TCR), respectively.
Results: I n treatment of BDC2.5 NOD mice with CTA1R7K-PS3-DD reduced proliferation and IFN-γ production to *in vitro* PS3 peptide stimulation. Transfer of CD4$^+$ BDC2.5 T cells to NOD.*scid* mice results in T1D development in 80–100% of recipient mice. In contrast, 95% of recipients of cells from BDC2.5 NOD mice treated with the CTA1R7K-DD peptide construct remained healthy. The i n treatment resulted in systemic increase in the frequency of CD4$^+$ BDC2.5 transgenic T cells expressing FoxP3, most enhanced in draining mediastinal lymph nodes.
Conclusions: We demonstrate that i n treatment with CTA1R7K-pept-DD induced tolerance in peptide specific diabetogenic CD4$^+$ T cells, and resulted in an increased frequency of Treg cells. The data suggest that CTA1R7K-peptide-DD may prevent T1D development by the induction of peptide specific CD4$^+$ Treg cells.

P1689

Treg differentiation induces by the immunosuppressive enzyme IL4I1

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Purpose/Objective: IL4I1 is an L-phenylalanine oxidase which inhibits the TCRζ chain expression and the proliferation of T lymphocytes *in vitro* [Boulland ML *et al.* (2007) *Blood* 110(1):220–227]. IL4I1 is highly expressed by macrophages infiltrating tumors and tumor cells of some cancers, particularly different B-cell lymphomas [Carbonnelle-Puscian A *et al.* (2009) *Leukemia* 23(5):952–60]. We recently showed in a mouse model that IL4I1 inhibits the cytotoxic antitumor T-cell response [Lasoudris F *et al.* (2011) *European Journal of Immunology* 41:1629–38].
Materials and methods: To evaluate the hypothesis that IL4I1 could contribute to the enrichment of regulatory T cells (Treg) in tumors, we used a recombinant monocytic cell line expressing IL4I1 [Marquet J *et al.* (2010) *European Journal of Immunology* 40(9):2557–68] to stimulate naïve CD4+ T cells and studied the effect on T helper cell differentiation in the presence or absence of polarizing stimuli.
Results: The phenotypic analysis of the population obtained showed an enrichment in CD25^{high} FoxP3$^+$ Treg endowed with *in vitro* suppressive activity. The mechanisms involved and the phenotypic characteristics of these cells are currently under investigation.
Conclusions: Thus, IL4I1 may participate to tumor escape both via inhibition of effector T cell proliferation and functions and Treg enrichment in the tumor microenvironment.

P1690

Use of the quantitative DNA methylation assay to measure Treg frequencies in the ovarian cancer patients

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Purpose/Objective: The type of immune cells that are present within the tumor microenvironment can play a crucial role in the prognosis of patients. However, little is known about the dynamics of the tumor-infiltrating immune cells during disease progression. We studied the immune cells infiltrating the tumor tissues of ovarian cancer patients at different stages of disease.
Materials and methods: We used the flow cytometry for detection Th17 cells and a dominant population of FoxP3$^+$ Helios$^+$ activated regulatory T cells (Tregs) in disseminated tumors (stage III-IV).

So far, the most specific marker for human naturally occurring Treg cells is the demethylation in the TSDR region of the transcription factor FoxP3. To measure nTreg frequencies we used methyl sensitive real time PCR assay (MS-qPCR) that is strictly specific for nTreg cells.
Results: The early stages of development of ovarian carcinomas were characterized by a strong Th17 immune response. A population of naturally occuring Treg cells was measured. We found that the frequency of nTregs significantly increases during the progression of the ovarian cancer.
Conclusions: Our findings demonstrate for the first time an alteration in the tumor-infiltrating immune cell pattern during ovarian cancer development and cleary show a dynamic shift from an active anti-tumor immune response represented by the presence of effector T cells, which prevail over Tregs in the early stages of EOC, to a significant immune suppression in the advanced stages of disease.

Poster Session: Immuno-Reset

P1691

A synthetic peptide that restores the inhibitory CD31 signaling as first-in class multifunctional anti-inflammatory drug

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Purpose/Objective: In addition to adaptive immune cells, platelets and endothelial activation contributes to dissemination of chronic inflammatory diseases, such as multiple sclerosis. CD31, a homophilic ITIM receptor exerts a global regulatory function on leukocytes, endothelial cells and platelets, but it is rapidly lost by cleavage upon cell activation. The aim of this study was to evaluate the inhibitory potential of a 8aa retro-inverso CD31-agonist synthetic peptide (P8RI) on cell activation *in vitro* and on the clinical course of Experimental Autoimmune Encephalomyelitis (EAE) *in vivo*.
Materials and methods: The *in vitro* effect of the peptide (5–100 μM) was evaluated on the activation of Human T cells by cytometric analysis of calcium mobilization in fluo 3-AM labelled Jurkat T cells stimulated with anti-CD3 antibodies and F(ab')2 anti-Ig fragments. The effect of P8RI on expression of VCAM-1 in response to IFNγ/TNF$\gamma\alpha$ stimulation by endothelial cells (HMEC/D3 cells) by immunofluorescence microscopy and cytometry. Finally, the anti-inflammatory effect of P8RI on platelets was tested on the release of soluble P-selectin (sCD62P) and IL-1β in response to tissue factor. Curative potential of P8RI was assessed on C57Bl6 mice (female, 8 week-old) subjected to MOG-induced EAE and clinically examined daily. When the clinical score reached a value ≥ 1, mice were randomized to receive either P8RI (2.5 mg/kg/day, sc) or prednisone (5 mg/kg/day, sc), or the vehicle alone (PBS, vol/injection = 50 μl).
Results: P8RI significantly reduced calcium mobilization in TCR-stimulated Jurkat T cells. In addition, it reduced the expression of VCAM-1 on stimulated endothelial cells as well as the release of sCD62P and IL-1b by tissue factor-stimulated platelets. In the curative EAE model, body weight and blood cell count were not affected by either the P8RI ($n = 14$) or prednisone ($n = 14$) as compared to control mice ($n = 13$). The time course of the clinical score was significantly bent by P8RI ($P < 0.01$ versus PBS, MANOVA). Of note, the extent of protection was even greater that that observed with prednisone ($P < 0.05$) up to 12 days of follow-up.
Conclusions: P8RI could be the first-in-class immune-regulatory drug that acts by rescuing a physiologic regulatory mechanism that is common to key cells involved in chronic inflammatory diseases.

Due to the fact that it does suppress/deplete resting blood cells, nor blocks a given immune pathway, its use may carry significantly fewer side effects as compared to other biologicals such as lymphocyte and cytokine neutralizing antibodies or soluble receptors.

Poster Session: Immunotherapy for Cancer

P1692

A high throughput phenotypic screen for Cbl-b antagonists on primary human T-cells

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Purpose/Objective: Cbl-b is a well validated target for anti-tumor immune therapy. It functions in controlling T cell anergy and thereby contributes to tumor growth and metastasis. Cbl-b is a member of the E3 ubiquitin ligase family and mediates ubiquitin attachment to key signaling molecules in immune cells. The Cbl-b protein consists of several conserved domains that mediate the interaction with a high number of proteins. This, together with the large size of Cbl-b protein, poses a challenge for a robust biochemical screen. Cbl-b causes numerous unique effects in T and NK cells and thus allows a number of specific cellular functional assays for inhibition of Cbl-b. Therefore, Cbl-b is a prototypic target for a cellular, phenotypic screen. In order to identify Cbl-b antagonists, a set of approximately 80 000 compounds was screened. To this end, human PBMCs have been employed in a phenotypic assay measuring IL2 secretion.
Materials and methods: Human PBMCs were isolated from pre-characterized donors, stimulated with CD3- and CD28-antibodies and used to screen 80 000 lead like compounds of Evotec's small molecule library at a concentration of 10 μM, phorbol ester stimulation was employed as a positive control. After overnight incubation, tissue culture supernatants were harvested and used to determine IL2 content by HTRF.
Results: Primary human PBMCs were obtained from pre-characterized volunteer donors. In order to identify Cbl-b antagonists, a phenotypic read-out was employed: Cells were stimulated with CD3/CD28 antibodies and Interleukin 2 content was measured after overnight incubation. Compounds that increased IL2 secretion in stimulated cells were selected. Counter screening was performed in the absence of cells, in order to rule out HTRF-related artifacts, and with naïve, non stimulated cells, to rule out pathway-unrelated stimulation of IL2 secretion. Screening of 80 000 substances revealed 39 confirmed, specific hits. The hit substances were clustered and revealed three different compound series in addition to numerous singleton hits. Hit expansion was performed by 2D based nearest neighbour searches and ca. One-sixty compounds together with the original hits were selected to be tested in 11 point concentration titration experiments on purified CD4+ T-cells.
Conclusions: Disease relevant, primary human T cells have successfully been employed in HTS using phenotypic, non-engineered read-outs and relevant chemical matter has been identified.

P1693

A mass spectrometry approach to identify HPV16 T cell epitopes for therapeutic vaccine design

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Purpose/Objective: To rationally design therapeutic HPV vaccines it is important to know which T cell epitopes are present on HPV-transformed malignant cells. HPV affects the cellular antigen processing machinery, thus not every epitope derived from viral proteins is necessarily presented by MHC molecules on the cancer cell surface.

Human papillomavirus (HPV) 16 has been identified as the causative agent in 50% of all cervical cancer cases and in virtually all extra-cervical mucosal HPV-induced tumors. The transforming potential of high-risk HPVs is mediated by two consistently expressed viral oncoproteins, E6 and E7. As the induction and maintenance of the malignant phenotype depends on these two proteins, they are ideal targets for immunotherapy. A therapeutic vaccine that is applicable to everyone without prior HLA typing needs to contain epitopes for all HLA types. Definition of epitopes for the five major HLA supertypes allows >95% population coverage.

To date, HPV T cell epitopes have mostly been determined by indirect methods and for the most prevalent HLA type, HLA-A2. In this study, we aim to directly identify HPV16 E6 and E7 T cell epitopes for the HLA-A3/HLA-A11 and HLA-A24 supertypes by a mass spectrometry (MS) approach.
Materials and methods: Several web-based prediction algorithms were used to predict prospective epitopes from the HPV16 E6 and E7 proteins for the above mentioned HLA supertypes. These candidate epitopes were tested for actual HLA binding in competition-based cellular binding assays. The presence of binding peptides on HPV16-transformed cells was analyzed by nano-UPLC-ESI-MS^2 and -MS^3 of immunoprecipitated HLA-A2-peptide complexes.
Results: *In silico* prediction resulted in 48 candidate epitopes for HLA-A3, 86 for HLA-A11 and 66 for HLA-A24. The majority of predicted peptides were found to bind to their respective HLA allele. Several novel binding peptides derived from HPV16 E6 and E7 were identified. MS analysis was successfully established and validated for HLA-A2.
Conclusions: We show that ascertaining the actual cellular presentation of HPV T cell epitopes is feasible. Having shown proof-of-concept for this approach for HLA-A2, it will now be extended to other HLA supertypes. Verified epitopes are the basis of rational therapeutic vaccine design and also important for immunomonitoring purposes.

P1696

A toxoplasma gondii vaccine strain transforms immunosuppressive ovarian cancer dendritic cells into immunostimulatory cells that trigger therapeutic antitumor immunity

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Purpose/Objective: Targeting tumor-associated immunosuppression may be necessary to stimulate effective therapeutic immunity against lethal epithelial tumors such as ovarian cancer, one of the most aggressive and most frequently occurring epithelial cancers. We examined efficacy and mechanisms of *Toxoplasma gondii* vaccine immunotherapy targeting $CD11c^+$ dendritic cells that exhibit proangiogenic and immunosuppressive activity and are the most abundant leukocyte present in the ovarian carcinoma microenvironment.
Materials and methods: Attenuated nonreplicating *T. gondii*; transplantable ID8-*Defb29/Vegf-A* and ID8-*Vegf-A* tumors; knockout mice; FACS analysis; cytokine analysis; tumor microenviroment.
Results: We show that an avirulent nonreplicating vaccine strain (*cps*) of *Toxoplasma gondii* preferentially targets and invades immunosuppressive $CD11c^+$ dendritic cells in the ovarian carcinoma microenvironment. Tumor $CD11c^+$ dendritic cells actively invaded by *cps* were activated into immunostimulatory phenotypes that expressed markedly increased levels of co-stimulatory molecules CD80 and CD86. In response to *cps* treatment of the immunosuppressive ovarian tumor environment, IL-12 was rapidly stimulated and antigen-presenting cells regained the ability to efficiently process and present antigen. Treatment of an extremely aggressive transplantable ovarian carcinoma (ID8-*Defb29/Vegf-A*) with *cps* provided a significant therapeutic survival benefit, and remarkably, *cps* treatment induced the rejection of a lethal aggressive model (ID8-*Vegf-A*) of established ovarian carcinoma. The therapeutic benefit of *cps* treatment was dependent on expression of IL-12 and interferon gamma. Surprisingly, the thera-

peutic effect was independent of MyD88 signaling as well as immune status to *Toxoplasma gondii*.
Conclusions: Our results establish that *cps* preferentially targets and activates tumor-associated dendritic cells to trigger the stimulation of potent antitumor responses. Immunotherapy based on *cps* reawakens natural immunity in immunosuppressive tumor environments and provides a new potent vaccine platform for engineering effective cancer vaccines based on the stimulation of tumor cell-specific $CD8^+$ T cells.

P1697
A tumor directed antibody_IL-15_IL-15Radomain fusionprotein for cancer immunotherapy

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Purpose/Objective: Cytokines driving the immune response are powerful tools for cancer immunotherapy, but their application is generally limited by severe systemic toxicity. Targeted approaches by means of antibody-cytokine fusion proteins might enable to focus the cytokine activity to the tumor site, thereby reducing unwanted side effects. Here we investigated the possibility to improve the efficiency of IL-15 presentation in a targeted approach by the incorporation of an IL-15Rα chain fragment, mimicking physiological trans-presentation. Therefore, an antibody cytokine fusion protein (scFv_RD_IL-15) composed of an antibody moiety (scFv) targeting the tumor stromal fibroblast activation protein (FAP), an extended IL-15Rαsushi domain (RD) and IL-15 and according control proteins devoid of one of the components were generated.
Materials and methods: FAP-transfected B16 melanoma cells were used as target cells for *in vitro* and *in vivo* experiments. Binding capacity of the scFv to FAP was analysed by flow cytometry. Cytokine activity of soluble and targeted fusion proteins was analyzed in terms of proliferation of cytokine growth-dependent cell lines (determined by MTT-assay) and human PBMC subsets (CFSE-staining/flow cytometry) and cytotoxicity of T cells (CD107a/flow cytometry). Analysis of pharmacokinetic properties and the therapeutic effect in a lung metastasis model were performed in C57/BL6 mice.
Results: All components of the fusion proteins showed full functionality: the scFv mediated specific binding to target-expressing cells and IL-15 stimulated the proliferation of cytokine-dependent cell lines and PBMCs. Soluble and target-bound scFv_RD_IL-15 achieved stronger proliferation and cytotoxicity of unstimulated and activated T cells, respectively compared to scFv_IL-15. Furthermore the scFv_RD_IL-15 revealed best anti tumor effects in a lung metastasis mouse model compared to an untargeted or RD missing version of the protein.
Conclusions: Thus, an antibody_RD_IL-15 fusion protein appears as a promising approach to further improve the antitumor effect of IL-15.

P1698
Activation of human dendritic cells for immunotherapy in ovarian carcinoma

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Purpose/Objective: In this study, we investigate the impact of ovarian carcinoma (OC)-associated ascites fluid (AF) on dendritic cell (DC) activation by Toll-like-receptor (TLR) agonists *in vitro*. DC have the potential to instigate a tumour-specific adaptive immune response, but their ability to induce differentiation of naïve lymphocytes into effector cells in lymphoid tissues is dependent on their activation status. We are interested in studying whether DC activation by TLR agonists * an approach often attempted in tumour immunotherapeutic concepts * is impeded by OC environment and if so, how these effects can be eleviated.
Materials and methods: AF was obtained from patients with epithelial OC or benign ovarian tumours with informed consent. Monocyte-derived DC from healthy volunteers were cultured overnight in the absence or presence of R848, LPS or poly I:C and AF. To investigate the role of different AF components, cytokine-neutralizing antibodies were added to selected cultures. DC activation was assessed by surface marker expression and cytokine production. Mixed leukocyte reactions with allogeneic naïve T-cells were performed to evaluate DC functionality.
Results: Our results show that AF reduces the up-regulation of the co-stimulatory molecule CD86 and partially inhibits the production of the pro-inflammatory cytokines IL-6, IL-12 and TNF-a in TLR-activated monocyte-derived DC. We further observe an impaired T-cell stimulatory capacity of monocyte-derived DC upon activation with TLR agonists in the presence of AF, which indicates that suppressed activation status of the monocyte-derived DC directly affects their function as antigen-presenting cells. We have identified IL-10 as the pivotal component in AF jeopardizing DC activation, and although further factors may play a role, our data show that selective blocking of IL-10 by neutralizing antibodies is sufficient to largely restore DC activation and function *in vitro*.
Conclusions: IL-10 is the key agent impeding TLR-mediated DC activation within OC environment *in vitro*. By selective neutraliziation, the immunosuppressive effects of IL-10 can be offset and DC functionality reinstated. Our findings can provide substantial insight into the feasibility and execution of DC-based vaccines and other immunotherapeutic intervention strategies in OC.

P1699
Actively personalized multi-peptide vaccination for primary liver cancers – a novel therapeutic approach for overcoming residual disease?

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Purpose/Objective: Primary liver cancers, mainly hepatocellular carcinomas (HCC), are among the five most common cancers globally and a leading cause of cancer related death. More than half a million patients are diagnosed with HCC every year. Also in Europe and the USA incidence numbers have been rising over the last decades.

With chemotherapies and other adjuvant strategies showing only limited benefit, available therapeutic options are scarce. Therefore primary malignancies of the liver are a promising entity for targeted immune interventions after surgery.
Materials and methods: In order to evaluate this novel targeted adjuvant therapeutic approach, we focused on naturally processed and presented MHC-ligands eluted directly from tumor tissue. Our interest is particularly centered on mutated peptides representing neo-epitopes derived from specific mutations of the tumor exclusively present on malignant tissue. Those tumor specific peptides lack central tolerance and should thus be highly immunogenic. To identify those peptides we employ a dual approach.

On the one hand MHC-ligands are isolated by means of immunoaffinity chromatography of MHC molecules followed by acidic

elution of peptides. These peptides are subsequently identified using tandem mass spectrometry and database dependent search algorithms. On the other hand genomic mutations forming the basis for tumor specific MHC-ligands are analyzed by next generation exome sequencing. Based on sequencing results differences between tumor and germline genome are determined and specific databases are generated.
Results: Tumor specific peptides are predicted using SYFPEITHI database to facilitate a targeted search for mutated peptides in the MS^2-data. In addition to tumor tissue adjacent benign tissue of patients was analyzed in the same fashion. So far sample pairs from several patients have been investigated in this manner.
Conclusions: Based on this approach, we aim at providing an actively personalized vaccine cocktail comprising individual patient- and tumor-specific peptides for adjuvant tumor therapy. This approach is completely new and may offer the chance of overcoming residual disease, which is a major cause of tumor related death and relapse so far.

P1701

Adoptive immunotherapy utilizing genetically modified T cells in an orthotopic breast cancer model

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Purpose/Objective: Adoptive immunotherapy involving genetic modification of T cells with chimeric antigen receptors (CARs) is a promising approach for the treatment of cancer. We have recently shown that the anti-tumour effects observed in Her-2 transgenic mice bearing established Her-2^+ lung metastases, treated with anti-Her-2 T cells was not associated with any autoimmune pathology to normal tissue (breast, brain) expressing the target antigen. However, the efficacy and safety of this approach against tumor located directly within normal tissue expressing the Her-2 antigen has not been evaluated.
Materials and methods: We generated a spontaneously metastatic breast carcinoma cell line, E0771-LMC expressing the human Her-2 antigen which displayed comparable growth kinetics of primary tumor and formation of spontaneous lung metastases as the parental cells following orthotopic injection into the mammary fat pad (MFP). We then examined antigen-specific function by CAR T cells by investigating cytokine secretion, killing and proliferation *in vitro*. In adoptive transfer studies, primary tumor growth and survival of mice was evaluated in the Her-2 transgenic mouse model.
Results: We first demonstrated antigen-specific function by CAR T cells *in vitro*. In adoptive transfer studies, we demonstrated significant regression of established primary Her-2^+ tumours injected into the MFP and enhanced survival of mice treated with anti-Her-2 T cells compared to control T cells. Increasing the dose of anti-Her-2 T cells led to complete tumour eradication in a proportion of mice. The reduction in tumour growth correlated with localisation of transferred T cells at the tumour site. Interestingly, we observed no overt toxicity in mice following therapy. Future studies will examine the efficacy of anti-Her-2 T cells against spontaneous metastases in this model and examine more closely breast and brain tissue by immunohistochemistry for potential signs of pathology.
Conclusions: In conclusion, our study highlights for the first time that CAR T cells can distinguish and specifically eradicate Her-2^+ breast cancer cells without on target toxicity to surrounding normal tissue expressing the target antigen.

P1702

Anti-CD20 treatment induces long-term adaptive immune response by blocking $CD4^+$ $Foxp3^+$ Treg expansion and provoking an increase in $CD4^+$ IFNgamma$^+$ Th1 cell subset

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Purpose/Objective: We have recently demonstrated that anti-CD20 mAb exerts a long-lasting anti-tumor protection through the induction of an adaptive immune response (Abes *et al.*, Blood, 2010). This requires the presence of $CD4^+$ cells at the initiation of treatment but not $CD8^+$ cells, whereas both cell types are needed after tumor challenge for further protection. It is unclear how anti-CD20 mAb treatment impacts $CD4^+$ T cell subsets, enabling the development of this adaptive response despite the fact that tumor cells induce regulatory T cells (Treg). Thus, we have investigated if anti-CD20 mAb treatment modifies the balance between T cell subsets and has an impact on the expansion of Treg induced by tumor cells.
Materials and methods: C57Bl/6 mice were injected with EL4-huCD20 cells and treated with the anti-CD20 mAb CAT-13 (Abes *et al.*, 2010). $CD3^+CD4^+$ T cell subsets were analyzed by intracellular staining with anti-IFNγ, -IL10, -IL17, and -Foxp3 antibodies. The log-rank test was used to compare survival. In some experiments, Treg were depleted by anti-CD25 injection. In other experiments, a neutralizing anti-TGFβ antibody was injected repeatedly.
Results: Adoptive transfer of $CD4^+$ T cells from CAT-13-treated surviving mice into naïve recipients, followed by EL4-huCD20 injection, showed that $CD4^+$ T cells are responsible for the induction of the long-term protection. Analysis of $CD4^+$ T cells, at different time points after tumor cell injection, initially revealed the expansion of Treg in both untreated and CAT-13-treated mice. However, after completion of anti-CD20 treatment, CAT-13-treated mice exhibited a strong Th1 subset increase while Treg diminished markedly. When Treg were depleted by anti-CD25 treatment before tumor injection, the survival of CAT-13-treated mice was not increased, although most of the mice not treated with CAT-13 were protected. This suggests that combination of Treg depletion before tumor injection with anti-CD20 treatment does not potentiate anti-tumor protection. Finally, injections of neutralizing anti-TGFβ did not increase survival of CAT-13-treated animals, although it delayed death of untreated animals.
Conclusions: These data demonstrate that the balance between Treg and Th1 compartments, essential for controlling tumor progression, is fine-tuned by therapeutic mAb to switch the immune response from a pro- to an anti-tumor response.

P1703

Anti-IL-20 monoclonal antibody alleviates inflammation within oral cancer and suppresses tumor growth

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Purpose/Objective: IL-20 is a proinflammatory cytokine involved in rheumatoid arthritis, atherosclerosis, and osteoporosis. However, little is known about the role of IL-20 in oral cancer.
Materials and methods: We explored the function of IL-20 in tumor progression of oral cancer. IL-20 expression levels in tumorous and non-tumorous oral tissue specimens from 40 patients with four different stages oral cancer were analyzed with IHC staining and RTQ-PCR. Clinical oral tumor tissue expressed higher IL-20 and its receptor subunits than did non-tumorous oral tissue. The roles of IL-20 were examined in oral cancer OEC-M1 and OC-3 cells.
Results: *In vitro,* IL-20 promoted TNF-a, IL-1b, MCP-1, CCR4, and CXCR4, and enhanced proliferation, migration, ROS production, and

colony formation of oral cancer cells with activated STAT3 and AKT/JNK/ERK signals. To evaluate the therapeutic potential of anti-IL-20 monoclonal antibody 7E to treat oral cancer, an *ex vivo* tumor growth model was used. *In vivo*, 7E reduced tumor growth and inflammation within oral cancer cells.
Conclusions: IL-20 promoted oral tumor growth, migration, and tumor-associated inflammation. Therefore, IL-20 may be a novel target for treating oral cancer, and anti-IL-20 monoclonal antibody 7E may be a feasible therapeutic.

P1704
Anti-tumoral effects of anti-CD115 monoclonal antibody through inhibition of tumor associated macrophages and osteoclasts

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Purpose/Objective: Some aspects of tumor progression are controlled by Tumor-Associated Macrophages (TAMs) and metastasis-induced bone destruction by osteoclasts. Both cell types depend on the CD115-CSF-1 pathway for their differentiation and function. We studied the effects of targeting CD115 with a function-blocking monoclonal antibody (mAb) in 3 different mouse cancer models.
Materials and methods: Anti-CD115 mAb, isotype control or PBS was administered to (1) mice bearing sub-cutaneous EL4 tumors, (2) MMTV-PyMT mice developing spontaneous mammary tumors, and (3) mice injected with human breast cancer cells metastatic to bone.
Results: In mice bearing solid EL4 tumors, the anti-CD115 mAb depleted F4/80 macrophages and reduced tumor growth, resulting in prolonged mouse survival. In MMTV-PyMT mice, primary tumor growth was not detectable in mice treated with the anti CD115 mAb but became palpable only after termination of the immunotherapy. In the breast cancer model of bone metastasis, the anti CD115 mAb showed potent therapeutic effect by inhibiting the differentiation of osteoclasts and their bone destruction activity.
Conclusions: CD115 represents a promising target for cancer immunotherapy, since a blocking antibody may not only inhibit the growth of a primary tumor through TAM depletion, but also metastasis-induced bone destruction through osteoclast inhibition.

P1706
Azacytidine treatment sensitize tumour cells to T-cell mediated cytotoxicity in patients with myeloid malignancies

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Purpose/Objective: We investigated a potential immunological effect of azacytidine (AZA) treatment in patients with myeloid malignancies, following the hypotheses that AZA-treatment may induce T-cell recognition of tumour-cells.
Materials and methods: Cytotoxicity of T cells was analysed by CD107a expression in co-cultures of purified tumour cells and CD8 T cells. Evaluation of CTA specific T cells was done using combinatorial encoded MHC-multimers after 7 days peptide restimulation.
Results: We could, in one patient, detect enhanced direct ex-vivo T-cell cytotoxicity against tumour-cells during treatment with AZA. Interestingly, tumour-cells were better recognized at later time-points during treatment, while T-cell functionality seemed to decline. In a larger group of patients, specific T-cell recognition of Cancer-Testis Antigens (CTA) was detected using a panel of 43 known CTAs.
Conclusions: Our data supports the hypotheses that AZA-treatment has an additional immunological effect that could be boosted in combination with immunotherapy.

P1709
Cancer immunotherapy by adoptive transfer of tumor-specific Th2 cells

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Purpose/Objective: Adoptive transfer of tumor-specific T lymphocytes is a promising strategy to cure cancer.
Materials and methods: We tested adoptive transfer of tumor-specific Th2 cells for the treatment of mice with major histocompatiblity complex (MHC) class II-negative MOPC315 myeloma.
Results: Transfer of tumor-specific Th2 cells efficiently eradicated myeloma and conferred long-lasting immunity. Tumor-specific Th2 cells produced interleukin-4 (IL-4), IL-5, and IL-13, and kept a stable Th2 phenotype *in vivo* upon transfer. Successful Th2-based immunotherapy did not require B cells, natural killer (NK) T cells, $CD8^+$ T cells, or interferon-γ. Upon transfer, tumor-specific Th2 cells were shown to induce an inflammatory reaction at the tumor site. Tumor-specific Th2 cells eradicated myeloma by rendering tumor-infiltrating M2-type macrophages tumoricidal.
Conclusions: These results suggest that adoptive transfer of tumor-specific Th2 cells could be a very efficient treatment against multiple myeloma and other human malignancies.

P1710
CD28 aptamers as immune response modulators

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Purpose/Objective: CD28 is one of the main costimulatory receptors responsible for the proper activation of T lymphocytes. Agonistic antibodies and recombinant-protein blockers for the CD28 receptor have already been identified and shown to alter the immune response. Soluble protein therapeutic molecules derived from cell-based procedures need to pass through a lot of high-cost regulatory processes to get into the clinic. Aptamers are single-strand oligonucleotides that exhibit similar affinity and specificity to that of an antibody and its ligand. Aptamers can easily be chemically synthesized, therefore reducing the cost of the clinical regulatory approval process.
Materials and methods: In this study, two aptamers to the CD28 receptor were selected by SELEX (Systematic Evolution of Ligands by Exponential Enrichment). *In vitro* immunoassays were performed to characterize the immunomodulatory aptamers. The agonistic CD28 aptamer was tested as an adjuvant in a mouse idiotypic vaccine protocol for follicular lymphoma.
Results: We have isolated and characterized two aptamers that bind to the CD28 receptor. One of them interferes with the binding of CD28 to its ligand (B7), precluding the costimulatory signal. *Vice versa*, dimerization of any of the two anti-CD28 aptamers is enough to

provide an artificial costimulatory signal. Different dimer structures were tested, and they have shown different costimulatory properties. In a follicular lymphoma model, the agonistic CD28 aptamer enhances the antitumor immune response of the idiotypic vaccine.
Conclusions: The CD28 aptamers described could be used to modulate the immune response whether by blocking the interaction with B7 for certain autoimmune diseases or by enhancing the vaccine-induced immune response in cancer immunotherapy.

P1711
CD4 cells expressing an MHC class I restricted TCR can rescue CD8 cells tolerized to tumour-associated antigen

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Purpose/Objective: The efficacy of T cell therapies for cancer may be limited because tumour-associated antigens (TAA) are also self-antigens (Ag). Exposure to TAA on normal cells may lead to tolerance via deletion of Ag-specific T cells or their anergy/exhaustion. We designed a model of tolerance to TAA in which T cell receptor (TCR)-transduced CD8 T cells recognise pMDM2, a TAA that is also a ubiquitous self-Ag. We aimed to determine the mechanism of tolerance and whether this is reversed by CD4 help.
Materials and methods: CD8 T cells transduced with pMDM2-specific TCR (MDM-CD8) were transferred to sub-lethally irradiated B6 mice expressing cognate MDM2 antigen (MDM100:K^b). MDM-CD8 cells were traceable using a congenic marker. To assess their function we transferred CFSE-labelled target cells pulsed with MDM2 peptide and compared killing at 16 h to that of a control population.
Results: Four weeks after transfer, MDM-CD8 cells were detectable, but showed defective Ag-specific killing of target cells.

To determine whether tolerance was dependent upon recognition of cognate Ag within the host, we transferred MDM-CD8 cells into BALBc mice (where MDM100:K^b can not be expressed). In a host environment free of cognate Ag, they retained a full capacity to kill target cells presenting Ag.

To determine the effect of CD4 T cell help, we co-transferred MDM-CD8 cells and transgenic OT-II CD4+ cells. OT-II cells were primed with dendritic cells (DCs) loaded with cognate pOVA323-339 or irrelevant peptide. After activation through their TCR, OT-II cells increased both the frequency of MDM-CD8 cells and their cytotoxic functions, indicating that CD4 help can overcome CD8 tolerance to TAA.

A major limitation to providing CD4 help in T cell cancer therapies is a lack of known MHC class II-restricted TAA. We therefore tested whether CD4 cells transduced with the MHC Class I-restricted MDM2 TCR (MDM-CD4) could provide help upon transfer to Ag-expressing hosts. Co-transfer of MDM-CD4 along with MDM-CD8 cells improved Ag-specific killing of target cells when compared to either population alone.
Conclusions: CD4 cells rendered capable of responding to an MHC class I-restricted TAA by TCR transfer can rescue tolerance in a CD8 population with the same specificity. This is potentially a novel way to circumvent defective immune responses arising in adoptive populations due to prolonged exposure to TAA.

P1713
Chemoimmunotherapy of MC38 mouse colon carcinoma: effect of cyclophosphamide treatment followed by dendritic cell-based vaccines on induction of anti-tumor immunity

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Purpose/Objective: The aim of this study was to analyze the effect of cyclophosphamide (CY) administration followed by bone marrow-derived dendritic cells (BM-DC) activated *ex vivo* with tumor antigens and applied with genetically modified dendritic cells of line JAWS II on trigger the antitumor response.
Materials and methods: Vaccines consisted of BM-DC activated with tumor lysate (BMDC/TAg) and IL-2-gene transduced JAWS II (JAWS II/IL-2) cells orneo-gene modified JAWS II/Neoused as a control. C57BL/6 mice were inoculated s. c. with MC38/0 colon carcinoma cells and on the 14thday, when tumors were palpable, mice were injected, i. p., with CY (150 mg/kg body weight). Starting on the 17thday, the cell vaccines were injected p.t., in 3 weekly intervals. Tumor growth delay was estimated. Spleens were obtained 1 week after the last injection. Splenocytes obtained from mice treated with CY +/- vaccines containing genetically modified cells or BM-DC/TAg cells were examined for their capacity of responding against growing tumor. Thus, the phenotype of freshly isolated cells as well as their *ex vivo* reactivity after TAg restimulation were analyzed.
Results: The administration of the CY combination with BM-DC/TAg and/or JAWS II/IL-2 strongly enhanced the effect of the cytostatic used alone. By contrast, the use of JAWS II/Neo cells caused no therapeutic effect. The combined therapy exploiting genetically modified DCs resulted in increase in percentage of $CD8^+$, $CD4^+$ and $CD49b^+$ cells. The 5-day cocultivation of splenocytes with MC38/0 cells induced cytotoxic activity to some extent associated with increase in percentage of $CD107a^+$ cells, augmented IFN-γ production and was accompanied by changes in percentage of $CD8^+$, $CD4^+$, $CD49b^+$ activated lymphocytes. The effect depended on the type of applied vaccines.
Conclusions: The obtained results suggest the strong augmentation of cytostatic effect by application of DC-based vaccines and may help to understand the mechanisms of the anti-tumor immune reactions initiated by combined chemoimmunotherapy of experimental murine tumors.

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P1714
Circulating specific antibodies enhance systemic cross-priming by delivery of complexed antigen to dendritic cells *in vivo*

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Purpose/Objective: DCs loaded with antigen-antibody complexes (ICs) have shown to confer tumor protection in mice. In the case of therapeutic antibodies directed to tumor-associated antigens (TAA), T cells may contribute to the induced anti-tumor response. To demonstrate the role of circulating antibodies in the priming of a naïve T cells, we applied a system in which mice, naïve for OVA, with circulating hapten (TNP)-specific antibodies were used.
Materials and methods: Mice were either vaccinated for a specific hapten (TNP) to induce hapten-specific antibodies, or passively transfered with the hapten-specific antibodies. These mice were then

injected with the TNP-OVA constructs to induce an immune response. The induction of an immune response was measure by *in vivo* antigen presentation assays, *in vivo* cytotoxicity assays and *in vivo* uptake experiments.
Results: Mice with circulating hapten-specific antibodies showed significantly enhanced cross-presentation of the injected antigen compared with mice that lacked these antibodies. Increased systemic CD8 and CD4 T cell proliferation was seen after TNP-OVA administration, demonstrating effective MHC class I and II (cross-) presentation. The enhanced cross-presentation in mice with circulating antigen-specific antibodies was associated with improved antigen capture by APCs. Importantly, CD11c+ APCs were responsible for the enhanced and sustained cross-presentation, although CD11c- APCs had initially captured a significant amount of the injected antigen. These results indicate that circulating antibodies aid *in vivo* antigen delivery to DCs, which improves vaccination by increased antigen uptake and FcR-mediated DC maturation.
Conclusions: *In vivo* formation of antigen*antibody immune complexes improves MHC class I cross-presentation, and CD8+ T-cell activation. This demonstrates that humoral immunity can aid the initiation of systemic cellular immunity. These findings have important implications for the understanding of the action of therapeutic antibodies against tumor-associated antigens intensively used in the clinic nowadays.

P1715
Combining a bispecific antibody with costimulatory antibody ligand fusion proteins in a human and murine model system for targeted cancer immunotherapy

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Purpose/Objective: Bispecific antibodies have shown to be able to retarget cytotoxic T cells to tumor cells in a MHC-independent manner, triggering effector cell activation and consecutive tumor cell killing. Considering that costimulation is an essential requirement not only to initiate T cell activation, but also for cell expansion and the development of T cell effector functions, we propose a combinatorial approach by a recombinant bispecific antibody and targeted costimulatory ligands of the B7 and TNF family in order to achieve a highly efficient antitumoral immune response. Therefore, appropriate model systems were generated that take into account targeting of different tumor antigens, combinations of costimulatory ligands and mouse or human effector cells.
Materials and methods: Immunostimulatory effects of the recombinant proteins (bispecific antibody + antibody-ligand fusion proteins) were assayed by incubation on tumor cells in presence of unstimulated human PBMC/isolated naïve CD8+ T cells or murine splenocytes. Cytokine release was monitored via ELISA, while proliferation (CFSE labeling), activation and differentiation marker expression and cytotoxic potential (CD107a staining) of T cells was detected via flow cytometry.
Results: Model systems involving tumor cells expressing 2 target antigens for the combined application of a bispecific antibody and costimulatory antibody-ligand fusion proteins with human and mouse specificity were established. In both experimental settings, bispecific antibody-induced T-cell stimulation could be enhanced by targeted costimulation with an antibody-4-1BBL fusion protein in a concentration-dependent and ligand-specific manner. Furthermore, in the human model system, modulation of T cell response by the application of the bispecific antibody together with two costimulatory fusion proteins (B7.2, 4-1BBL) resulted in enhanced proliferation and activation marker expression, differentiation into an activation-experienced memory phenotype and in an increase of cytotoxic potential.
Conclusions: In our model systems we could show that the combination of a bispecific antibody and costimulatory antibody-ligand fusion proteins appears as a promising strategy for cancer immunotherapy.

P1716
Controlled local delivery of CTLA-4 blocking antibody induces CD8+ T cell dependent tumor eradication without systemic toxicity

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Purpose/Objective: Blockade of CTLA-4 by monoclonal antibodies improves anti-tumor T cell responses in both pre-clinical models and clinical trials and is now approved for treatment of melanoma patients. However, treatment with CTLA-4 blocking antibodies is associated with auto-immune and inflammatory side-effects. In order to reduce side-effects we propose using a local administration for CTLA-4 blocking antibodies, previously shown to be very effective in CD40 agonistic antibody treatment of tumor.
Material and methods: We use an *in vivo* tumormodel to explore administration strategies of CTLA-4 blocking antibodies. By injecting the antibodies in a subcutaneous slow-release delivery formulation in the tumor area, we show that an eightfold lower dose of antibody is as effective in activating a tumor-eradicating T cell response as systemic delivery.
Results: Lower dose and slow release together result in 1000-fold decreased levels of antibody in the serum, reducing adverse events and the risk of auto-immunity. The main target and effector cells in this tumor-model are endogenous CD8+ T cells, whereas CD4+ T cells do not play a prominent role in the antibody-mediated tumor eradication.
Conclusions: These results call for exploration of a similar delivery principle in the clinic to reduce toxic side effects of CTLA-4 blocking antibody.

P1717
Culture expansion alters synthesis and content of actin binding proteins in T lymphocytes and mesenchymal stromal cells (MSCs)

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Purpose/Objective: The expansion of T lymphocytes and MSCs in culture is pursued for adoptive therapy, however deficits in the migration potential and function of expanded cells have been reported. We asked here whether major elements of the cytoskeleton, including actin binding proteins (APBs) such as the actin-polymerizing factors cofilin and profilin, small intercalating molecules filamin A and alpha actinin, as well as components engaging into focal adhesion complexes paxillin, talin and vinculin are subject to alterations during culture in these two cell types.
Materials and methods: Magnetically separated CD3+ T lymphocytes from peripheral blood were expanded using immobilized anti-CD3 and anti-CD28 microbeads. MSCs were expanded from human bone marrow according to standard protocols. Cell size was monitored microscopically and by flow cytometry, using size-calibrated micro beads. Quantitation of ABP RNA was performed using qRT-PCR and of ABP proteins by flow cytometry in permeabilized cells.
Results: T lymphocytes expanded 15 ± 1 fold within 7 days, but over the culture period, the mean diameter of T lymphocytes increased from 7 ± 0.3 to 15 ± 4.4 μm reflecting a ninefold increase in cell volume from a mean 208 to 1828 μm^3. However, on a per cell base, protein levels of ABPs remained constant except for vinculin, which showed a statistically significant 1.6-fold increase. In contrast, RNA levels of all investigated ABPs decreased between five and 50-fold

during culture expansion, indicating major alterations in ABP protein turnover in the expanded cells. MSCs revealed two cell populations with mean diameters of 38 ± 5 and 71 ± 9.6 μm, respectively. Compared to expanded T lymphocytes, MSCs showed a four to13-fold lower content of all investigated ABPs on both, the protein and mRNA levels.

Conclusions: In conclusion, culture expansion resulted in maintenance of absolute levels of ABPs in T lymphocytes, but drastic decreases in the relation between ABPs and cytoplasmic volume. The strong decrease in transcription of ABP mRNAs indicates major alterations in the turnover of ABPs protein in both T cells and MSCs. Our data point to role of alterations in ABP turnover in the functional deficits observed in cultured versus freshly isolated cells which are used for immunotherapies in patients.

P1719

Cytosolically targeted isRNA induces melanoma destruction via type I interferon signaling on myeloid cells in the tumor microenvironment

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Purpose/Objective: Activation of the ubiquitously expressed cytosolic RNA recognition receptors with immunostimulatory RNA (isRNA) emerges as a new treatment option for cancer. This mimics a viral infection and induces a strong type I interferon response.

Materials and methods: Here we investigated the cell type specific role of type I IFNs using Ifnar1 competent and deficient melanoma cell lines established from Hgf-Cdk4^{R24C} mice in combination with global and tissue-specific Ifnar1 knockout mice.

Results: pI:C complexed with polyethylenimine but not naked pI:C induces regression of primary cutaneous melanomas in Hgf-Cdk4^{R24C}. This was accompanied by a strong infiltration of immune cells mostly of myeloid origin.

In vitro, we observed similar chemokine release and apoptosis of pI:C-PEI treated Ifnar1 competent as well as deficient melanoma cells. In contrast, BM-derived Ifnar1$^{-/-}$macrophages and DCs showed an impaired chemokine release compared to wildtype cells after pI:C-PEI stimulation.

In vivo, pI:C-PEI had significant antitumor activity against transplanted Ifnar1-competent or -deficient melanoma cells in syngeneic wildtype but not in Ifnar1 knockout mice, demonstrating the importance of type I interferon signaling in the tumor microenvironment, but not in tumor cells.

Because myeloid cells were the predominant immune cell population infiltrating the tumor after pI:C-PEI therapy, we treated Ifnar1 competent melanoma cells in LysMCre × Ifnar1$^{fl/fl}$ mice, that lack the type I IFN receptor on myeloid cells. Again, treatment efficacy was largely abrogated, indicating that myeloid cells are the primary target of type I interferons. To further delineate if cytotoxic cells were required for tumor destruction, we depleted NK and CD8 T-cells with monoclonal antibodies. Whereas the elimination of NK cells strongly reduced treatment efficacy, the elimination of CD8 T-cells had no affect.

Conclusions: Taken together, our data suggest that melanoma therapy with cytosolically targeted immuno-stimulatory RNA largely depends on a functional type I IFN system in myeloid cells of the tumor microenvironment and the presence of NK cells.

P1720

Effective activity of cytokine-induced killer (CIK) cells against autologous putative cancer stem cells in solid tumors

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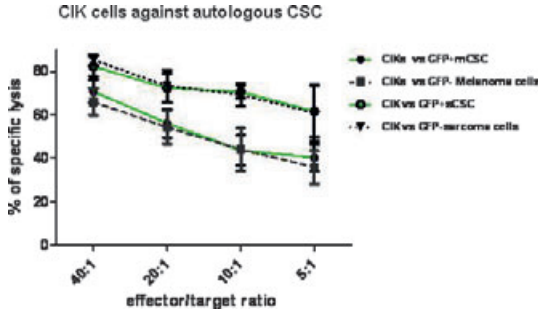
Purpose/Objective: Targeting the putative subset of cancer stem cells (CSC) within solid tumors is a crucial and still unmet issue as CSC are considered responsible for chemo-resistance and disease relapses. Cytokine-Induced Killer (CIK) Cells are ex-vivo expanded T lymphocytes, endowed with a promising MHC-independent tumor killing activity against several solid tumors.

We investigated the ability of CIK cells to kill autologous metastatic cells including the subset of putative CSC.

Materials and methods: CIK cells were expanded from PBMC of 10 patients with metastatic melanoma ($n = 5$) or bone and soft tissue sarcomas ($n = 5$); their tumor killing ability was assessed with standard cytotoxicity essays against autologous targets obtained from biopsies of metastatic samples. To identify putative CSC, we transduced bulk autologous metastatic tumor cells with a lentiviral vector encoding for the enhanced Green Fluorescent Protein (eGFP) regulated by the human OCT4 promoter. The underlying idea is that CSCs can be visualized based on their exclusive ability, proper of both normal and cancer stem cells, to activate the OCT4 promoter and consequently express eGFP.

Results: We efficiently generated metastatic autologous cells lines. The average presence of GFP+ putative melanoma CSC (mCSC) and sarcoma CSC (sCSC), within the bulk metastatic tumor cell population, was 12% and 15% respectively; these data were consistent with levels of protein expression. Both mCSC and sCSC displayed on average a 3 times reduced proliferative potential compared with their GFP- counterpart after 14 days of culture ($n = 6$), showing a slow-growing phenotype typical of CSC.

CIK cells efficiently killed both autologous mCSC and sCSC; the average specific killing was 71%, 57%, 44% and 41% against mCSC ($n = 4$), 82%, 72%, 71% and 60% against sCSC ($n = 4$) at 40:1, 20:1, 10:1 and 5:1 effector/target ratios respectively. The specific killing against CSC overlaid that observed against differentiated GFP- metastatic cells (Fig. 1).



Conclusions: We reported for the first time the preclinical activity of an immunotherapy approach with CIK cells against autologous CSC enriched fractions of metastatic melanomas and sarcomas. These data set biologic basis for further clinical investigations and picture CIK cells as promising candidates for effective immunotherapy in currently incurable clinical settings.

P1721

Elimination of MDSC and Treg cells after combined immunotherapy with cyclophosphamide and dendritic cell vaccines in MC38 tumor bearing mice

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Purpose/Objective: The microenvironment of solid tumors is characterized by excessive amounts of immunosuppressive factors e.g. VEGF, TGF-β, IL-10 which cause dysfunctions of immune competent cells as well as activation of suppressive cells. The tumor-infiltrating suppressive cells e.g. T regulatory cells (Treg), tumor associated macrophages and myeloid-derived suppressor cells (MDSCs) interact with one another and have an important impact on cancer disease progression. Recent work highlighted the immunostymulatory and anti-angiogenic effects of low dose of cyclophosphamide (CY). Thus, it could be successfully combined with new-generation anti-cancer vaccines.

The aim of the study was to estimate the anti-tumor effect of low dose of CY combined with dendritic cells stimulated with tumor antigens (BM-DC/TAg) and/or genetically modified dendritic cells producing IL-12.

Materials and methods: Mice with s.c. growing, advanced MC38 tumor were treated with CY followed by three consecutive injections of BM-DC-based vaccines. Tumors were measured every 2 days and the tumor growth inhibition was calculated. Seven days after the last injection of BM-DC based vaccines spleens and tumors were collected and the activation of systemic and local anti-tumor response were evaluated.

Results: It was observed that CY combined with BM-DC/TAg and/or BM-DC/IL-12 caused the highest tumor growth inhibition. Moreover, in these groups the immunohistological analysis of tumor tissues confirmed the most intensive influx of CD4+ and CD8+ lymphocytes into tumor. Restimulated *in vitro* spleen cells also showed increased anti-tumor activity. Spleen cells obtained from mice treated with CY and BM-DC/TAg and/or BM-DC/IL-12 showed the highest cytotoxic activity toward MC38 cells and among them the highest percentages of CD8+CD107a+ and CD49b+CD107a+ cells were detected. It was also observed that numbers of MDSC and Treg cells in spleens were different in all examined groups and decreased in mice treated with CY and BM-DC/TAg and/or BM-DC/IL-12.

Conclusions: Obtained results suggest that anti-tumor effect of chemoimmunotherapy with CY and DC-based vaccines is dependent on successful elimination of suppressor cells.

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P1722

Exploring novel therapeutic antibodies against c-Met

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Purpose/Objective: c-Met, a receptor tyrosine kinase overexpressed in many types of cancer, represents an attractive target for antibody therapy. Use of traditional bivalent IgG is often problematic due to agonistic activity of the antibody. Binding of bivalent IgG can induce dimerization of c-Met and induce downstream signaling, ultimately resulting in cell proliferation, invasion and survival. Here we present various strategies to circumvent the problem of agonism and we show *in vitro* and *in vivo* efficacy of novel antagonistic antibodies against c-Met.

Materials and methods: A diverse panel of human antibodies against c-Met was generates and characterized. The lead clones were subcloned in various monovalent and bivalent formats to determine the optimal format for c-Met targeting. The different antibody formats were tested in various *in vitro* assays including ligand inhibition, target down-modulation, receptor phosphorylation, proliferation and migration assays as well as *in vivo* xenograft models.

Results: We have generated a diverse antibody panel against c-Met including clones that could effectively block the ligand for c-Met and inhibit viability and growth of cells. Residual agonistic activity that was observed in some assays and conditions by bivalent IgG and could be modulated by either generating monovalent or engineering the backbone of the antibody to make the antibody more rigid. Efficacy in multiple cell lines both *in vitro* and *in vivo* models was shown with these engineered formats.

Conclusions: Here we present an overview of the required characteristics of therapeutic antibodies against c-Met with regard to epitope and antibody format. We were able to generate full c-Met antagonists by expressing antibodies in a monovalent format, without compromising the characteristics of the original IgG1 molecules. These novel antibodies have potential for successful targeting of c-Met expressing cancers.

P1723

Expression of ganglioside GM1 in osteosarcoma and Alzheimer's disease and its significance in B-cell functions

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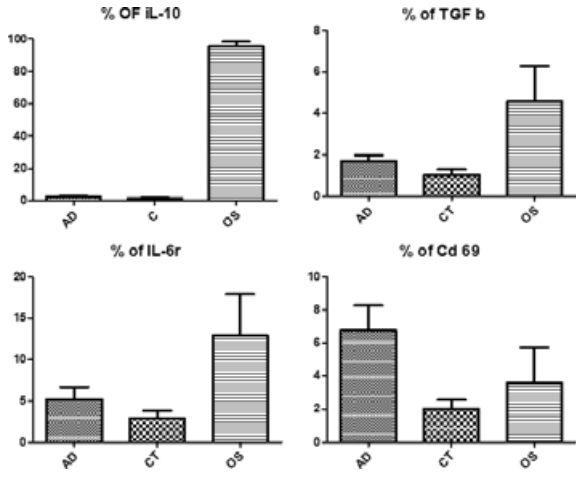
Purpose/Objective: Introduction: In the immune system GM1 is found on the surface of natural killer cells (NK), T cells (both CD4+ and CD8+), B cells, plasmocytes and monocytes. The very low incidence of cancer among the AD patients, has led us to study the differences in GM1-expression in various B lymphocyte subpopulations of osteosarcoma (OS) and AD patients, and whether they show any alterations in the expression of the cytokines and cell-surface markers.

Materials and methods: Methodology: Peripheral blood mononuclear cells were isolated from blood samples were collected from OS patients (before starting chemotherapy, $n = 6$), early stage AD patients



($n = 10$) and matched healthy controls (no infection or chronic inflammatory disease, $n = 10$). The GM1-expressing B lymphocytes were identified using FITC-labeled CTxB. The changes in cell surface markers expression and intracellular cytokine production were determined by flow cytometry. Data were analyzed using a non-parametric one way ANOVA, and differences were considered different for $P < 0.05$.

Cell populations	Mean ± SEM	*P* value	Median	MS
Il-10 − AD	2.59 ± 0.47	0.0011***	2.025	17730
CT	1.66 ± 0.28		1.430	
OS	95.81 ± 2.36		96.970	
Tgf-B − AD	1.71 ± 0.27	0.0057**	1.670	21.77
CT	1.02 ± 0.27		0.652	
OS	4.57 ± 1.72		3.720	
il-6r − AD	5.21 ± 1.45	0.0181*	3.915	169.6
CT	2.89 ± 0.93		1.601	
OS	12.89 ± 5.01		5.780	
Cd-69 − AD	6.77 ± 1.50	0.0355**	5.715	57.43
CT	2.03 ± 0.55		1.535	
OS	3.60 ± 2.15		0.150	
Plasmocytes-AD	1.16 ± 0.24	0.5164 (NS)	1.016	0.6752
CT	0.83 ± 0.40		0.233	
OS	0.54 ± 0.30		0.130	
Baff-r − AD	88.33 ± 1.70	0.0455*	88.550	177.4
CT	80.80 ± 2.69		90.350	
OS	78.83 ± 6.74		80.610	

AD, Alzeimer's Disease; Os, osteosarcoma; CT, controls; SE, standard error; MS, mean square; NS − not significant. *P* value < 0.05 [***, **. *].

Conclusions: Discussion: Our data showsignificantly higher levels of expression of GM1 on the surface of peripheral B cells of OS and AD patients versus Controls (OS > AD > Cnt). Moreover, our present data show, for the first time, that the increased levels of expression of IL-6R, IL-10, TGF *β on GM1+ B cells might link GM1-expression to B cell function, thus opening new avenues for therapeutic modulation of these cells in the context of cancer and neurodegeneration.

P1724

Fast 3 days poly (I:C)-activated dendritic cells generated in CellGro for use in cancer immunotherapy trials are fully comparable to standard 5 days DCs

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Purpose/Objective: Dendritic cells (DCs) are professional antigen-presenting cells capable of inducing immune responses. DC-based vaccines are normally generated using a standard 5−7 days protocol by culturing blood monocytes in the presence of cytokines IL-4 and GM-CSF. In order to shorten the vaccine production for the use in the cancer immunotherapy protocol and to generate DCs in GMP quality in shorter time we have developed fast DC protocol by comparing standard DCs (5 days) and fast DCs (3 days).
Materials and methods: In this study, we tested D5 versus D3 DCs generation using GMP culture media CellGro and subsequent activation by two activation stimuli * Poly (I:C) (TLR-3) and LPS (TLR-4). We evaluated DCs morphology, viability, phagocytic activity, cytokine production and ability to stimulate antigen specific T cells.
Results: Our results demonstrate that although D5 DCs were larger in size compared to D3 DCs, they exhibited similar phagocytic capacity. Viability of D5 and D3 DCs was comparable (80%). Poly (I:C) activated D5 DCs expressed higher levels of costimulatory and surface molecules CD80, CD86 and HLA-DR compared to Poly (I:C) activated D3 DCs. Nevertheless LPS activated D5 and D3 were phenotypically similar. The cytokine production was generally stronger when LPS was used as maturation stimuli. Surprisingly LPS activated D3 DCs were able to produce higher levels of IL-12p70 compared to D5 DCs. Furthermore Poly (I:C) activated D5 DCs secreted higher amounts of IL-6, IFN-α and TNF-α compared to D3 DCs. Importantly, Day-3 and Day-5 DCs were able to induce comparable numbers of antigen-specific T cells.
Conclusions: In this study, we identified monocyte-derived DCs generated in CellGro for 3 days and activated using Poly (I:C) as similarly potent clinical-grade DCs when compared to DCs produced by the standard 5 day protocol. These results provide the rationale for the testing of the faster protocols for DCs generation in the clinical trials. Generation of fast DCs markedly reduces the time, work load and cost associated with *in vitro* culture of DCs precursors and thus may facilitate the use of DCs in clinical trials of cellular immunotherapy.

P1725

Functionalized dendrimer for the *in vivo* specific genetic regulation of tumor immunity

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Purpose/Objective: While mature dendritic cells (DC) can support a strong tumor immunity leading to tumor immune surveillance or eradication, tumor conditioned myeloid cells (TCMC, aka Myeloid Derived Suppressor Cells, MDSC, and Tumor Associated Macrophage, TAM) promote tumor progression, invasion and metastasis. A specific modulation of these populations can be the key for the effective immune therapeutic treatment of malignacies. To boost tumor immunity while reversing TCMC tolerogenic program we used functionalized dendrimer to direct DNA encoding tumor antigen/s into immunogenic APC and STAT3 specific shRNA into suppressive TCMC. To this aim we generate two classes of nanoparticles in which a targeting peptide is covalently linked to G5 PAMAM dendrimers that serve as a loading surface for the desired nucleic acid. The first nanoparticle (HAPD) specifically target dendritic cells, the second one (4PD) instead deliver its cargo particularly to TCMC.
Materials and methods: MHC class II or IL4 derived targetting peptides were coupled to the dendrimer by maleidoamide chemistry and the complex purified by HPLC. Nucleic acids were loaded on the funcionalized dendrimer by electrostatic interaction at RT in <5 min in PBS. pcDNA3-gp70 conjugated to HAPD was used as targetted vaccine while STAT3 specific shRNA was used to modulate TCMC suppressive actions. The treatments were used in the colon carcinoma CT26 and mammary carcinoma TSA.
Results: The functionalized nanoplatforms were synthetyzed and characterized. Using either pcDNA3-GFP or Alexa555-shRNA their specificity was evaluated *in vitro* and confirmed *in vivo*. 4PD mediated STAT3 silencing in TCMC reduced the number of monocitic MDSC and increased the number of dendritic cells, promote tumor immunity, and significantly reduced tumor progression. HAPD mediated DNA vaccination against gp70 promote tumor immunity and delay tumor progression. Combination experiments are ongoing and will be presented.

Conclusions: By conjugating specific targeting peptides to G5-PA-MAM dendrimers we were able to modulate both the effector and the suppressive arm of the immune system. Our data indicate that the *in vivo* cell specific targeting of TCMC and DC can represent a new avenue for the immune therapeutic treatment of malignancies.

P1726
High throughput *in vitro* priming of tumor specific T cells

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Purpose/Objective: Peptide-based cancer vaccines are one promising strategy for immunotherapy against cancer. Our group has characterized large numbers of HLA ligands from tumor cells; among them many that might be used for *in vivo* induction of tumor-specific immune responses. For the development of peptide-based immunotherapies such peptides should be able to induce *in vivo* tumor-directed immune responses, especially by $CD8^+$ T cells that have a key role in inducing death of tumor cells. The aim of our work is to establish a fast and efficient workflow to induce tumor-reactive T cells which is based on immunogenicity testing of tumor-extracted HLA ligands.
Materials and methods: T cells usually depend on professional antigen presenting cells such as autologous dendritic cells (DCs) for specific induction and expansion. Since the generation of dendritic cells is expensive and time consuming with varying quality and amount of differentiated DCs, we established an artificial system to replace cell-based *in vitro* priming of T cells: streptavidin-coated artificial antigen presenting cells loaded with defined amounts of recombinant HLA molecules and costimulatory antibodies such as anti-CD28 and anti-CD137 (4-1BB).
Results: We tested abundant self-peptides with a substitution of one amino acid. The mutation is a part of the TCR binding site and not affecting the anchor positions. We identified peptide-specific $CD8^+$ T cells after *in vitro* priming of healthy HLA-matched blood donors. Specific T cells are reactive to mutated peptides but not to native peptides.
Conclusions: Employing artificial APCs without the need for autologous DCs enables successful *in vitro* priming of specific T cells. Our strategy promotes the opportunities of multiepitope vaccines in tumor immunotherapy.

P1727
HLA ligandome analysis of acute myeloid leukemia reveal novel tumor-associated antigens for immunotherapy

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Purpose/Objective: Acute myeloid leukemia (AML) is the most common acute leukemia in adults with an incidence rising with age. In the western world it is accounting for 25% of all leukemias. Owing to the therapeutic golden standard a remission is induced in 60−80% of all patients. However due to high relapse rates the 5 years survival ranges from 20% to 40%. To improve the overall survival the reduction or even eradication of remaining leukemia cells (minimal residual disease, MRD) by antigen-specific immunotherapy is a promising option. Therefore the major aim of this study is the identification of novel leukemia-associated, naturally presented HLA ligands (LEUKAPs) being able to induce a tumor specific T-cell response. Analyzing HLA class I ligands from primary AML-cells we have taken a special interest in peptides derived from AML-specific, mutated proteins.
Materials and methods: HLA class I ligands were isolated from patients' peripheral blood (blast count >80%) or PBMCs from healthy donors using immunoprecipitation. In order to identify HLA presented peptides, liquid chromatography coupled mass spectrometry (LC-MS/MS) based peptide sequencing was employed. The identified ligands were mined for leukemia associated peptides *via* comparing the HLA ligandomes of AML-patients with those of healthy donors, by analyzing gene expression databases and literature research. In order to identify veritable tumor specific peptides, acquired data has been mined for peptides derived from established leukemia associated mutations comprised in the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/).
Results: Having identified more than 6000 HLA ligands out of 6 AML patients, several new AML associated peptides (e.g. ligands derived from tumor protein p53, c-myc oncogene, KIS) were obtained by cross checking for occurrence on healthy tissues, especially on PBMCs. The sequences of identified LEUKAPs were verified by LC-MS/MS based peptide sequencing of their synthetic counterparts. Identified candidate peptides are currently checked for their immunogenicity after *in vitro* T cell priming.
Conclusions: This study provides several new leukemia associated antigens, for the first time directly obtained from AML patients' HLA ligandomes, offering promising options for a more efficient treatment of AML in the future.

P1728
HPMA copolymer-modified IL-2 possesses superior biological activity to free IL-2 *in vivo*

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Purpose/Objective: Interleukin-2 possesses strong stimulatory activity for activated T and NK cells and thus it is an attractive molecule for immunotherapy. However, its unfavourable pharmacological properties, extremely short half-life and severe toxicities associated with high-dose IL-2 are the most serious and limiting drawbacks. In order to increase IL-2 half-life and stability *in vivo*, we covalently conjugated IL-2 to synthetic semitelechelic polymeric carrier based on *N*-(2-Hydroxypropyl) methacrylamide (HPMA). Thus, we synthesized IL-2-pHPMA conjugate containing an average 2−3 polymer chains per IL-2 molecule.
Materials and methods: Expansion of CD8 ± T lymphocytes, NK and Treg cells *in vivo*. Purified OT-1 $CD8^+$ T cells (Ly5.2) were labeled with CFSE and injected i.v. into B6.SJL recipients (Ly5.1) on day 1. On day 2 the mice were injected i.p. with PBS, SIINFEKL peptide plus IL-2 (50 μg), and SIINFEKL peptide plus IL-2-pHPMA (1 μg of IL-2). Splenocytes were isolated day 7 and relative expansion of $Ly5.2^+$ $CD8^+$ T cells, NK cells, memory $CD8^+$ T cells and T regulatory cells was analyzed.

Kinetics of IL-2-pHPMA in blood. B6.SJL mice were injected i.v. with PBS, free IL-2 (2 μg) and IL-2-pHPMA (2 μg of IL-2). Blood sera were isolated at various time points after injection. Serum IL-2 concentrations were determined by sandwich ELISA with anti-mouse JES6.1A12 and anti-mouse JES6.5H4-biotin mAbs.

Determination of MTD. B6.SJL mice were injected i.p. with PBS or IL-2-pHPMA (25, 50 and 75 μg of IL-2) on day 0 . Weight of each mouse was recorded prior to injection (day -1) and for 6 days after. Average % weight change for each group was plotted.
Results: IL-2-pHPMA conjugate was found to be significantly more potent than free IL-2 in terms of expansion of NK, NKT, $\gamma\delta$T, memory

$CD8^+$ T and also T regulatory cells *in vivo*. IL-2-pHPMA conjugate also potently expanded adoptively transferred OT-1 $CD8^+$ T cells activated with SIINFEKL peptide while free IL-2 at the same dosage (1 μg IL-2 i.p. daily for 4 days) showed minimal activity. Moreover, activated naïve OT-1 $CD8^+$ T cells expanded by IL-2-pHPMA conjugate established a robust population of long-lived cells with memory phenotype, which were able to express effector functions upon re-activation. We also determined IL-2-pHPMA half-life in circulation upon intravenous administration. We found that short half-life of IL-2 is greatly prolonged by modification of IL-2 with HPMA polymeric carrier. Finally, we determined maximal tolerated dose of IL-2-pHPMA which is about 80 μg in case of single i.v. administration.
Conclusions: IL-2-pHPMA conjugate showed much longer biological half-time (~4 h versus <15 min) and superior biological activity than free IL-2 *in vivo*.

P1729

Human $CD8^+$ T cells engineered with supraphysiological TCRs are functionally inhibited via PD-1 and SHP-1 phosphatase

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Purpose/Objective: Protective $CD8^+$ T cell responses rely on TCR-dependent recognition of immunogenic peptides presented by MHC I. Cytolytic T lymphocytes directed against self/tumor antigens express TCRs of lower affinity/avidity than pathogen-derived T lymphocytes and elicit less protective immune responses due to mechanisms of central and peripheral tolerance. Anti-tumor T cell reactivity can be improved by increasing the TCR-pMHC affinity within physiological limits, while intriguingly further increase in the supraphysiological range (K_D < 1 μM) leads to drastic functional declines.

We aim at identifying the molecular mechanisms underlying the loss of T cell responsiveness associated with supraphysiological TCR-pMHC affinities in order to improve effectiveness of TCR-engineered T cells used in adoptive cell transfer (ACT) cancer immunotherapy.
Materials and methods: Using a panel of human $CD8^+$ T cells engineered with TCRs of incremental affinity for the HLA-A2-resticted tumor cancer testis antigen NY-ESO-1, we performed comparative gene expression microarray and TCR-mediated signaling analysis together with membrane receptors level analysis.
Results: As compared to cells expressing TCR affinities generating optimal function (K_D from 5to 1 μM), those with supraphysiological affinity (K_D from 1 μM to 15 nM) had an overall reduced expression of genes implied in signaling, cell activation and proliferation, and showed impaired proximal and distal TCR signaling capacity. This correlated with a decline in surface expression of CD8b, CD28 and activatory TNFR superfamily members. Importantly, expression of inhibitory receptor PD-1 and SHP-1 phosphatase was upregulated in a TCR affinity-dependent manner. Consequently, PD-L1 and SHP-1 blockade restored the function of T cells with high TCRs affinity. Moreover, SHP-1 inhibition also augmented functional efficacy of T cells with TCRs of optimal affinity.
Conclusions: Our findings indicate that TCR affinity-associated regulatory mechanisms control T cells responsiveness at various levels to limit potential auto-reactive cytotoxic effects. They also support the development of ACT therapies combined with blockade of inhibitory molecules such as SHP-1 to enhance effectiveness of T cell immunotherapy.

P1730

Identification of naturally presented tumor derived HLA Ligands — separating tumor and stroma origin

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Purpose/Objective: Renal cell carcinoma and ovarian cancer are both characterized by poor prognosis and high mortality due to late diagnosis and high resistance to conventional chemo- and radiotherapy. Looking for new and effective therapeutic options a lot of effort has been put into the development of a specific immunotherapy, aiming at the *in vivo* induction of a tumor-directed immune response. Our group is especially interested in the identification of tumor specific MHC ligands that can be used for the development of a multivalent peptide vaccine. For several years we have mainly relied on bulk tumor tissue to perform HLA ligand extraction and analysis by mass spectrometry. However information about the amount of isolated MHC molecules as well as bound peptides directly derived from tumor cells has so far never been obtained.
Materials and methods: In order to get a comprehensive view of the distribution of MHC expression within tumor tissue we used multi color flow cytometry to analyze the cellular composition and quantified the MHC expression of specific cell subsets. Single cell suspensions of fresh tissue samples were prepared by enzymatic digestion and stained with respective antibodies to distinguish between tumor cells and stroma (leukocytes, endothelial cells and fibroblasts). In a second step we further wanted to compare the overlap of identified HLA ligands directly on the peptide level. Therefore we started again from a bulk tissue derived single cell suspension but this time used MACS-technology to separate tumor from stroma cells before isolating and identifiying HLA ligands.
Results: Our results show that MHC molecules expressed by tumor cells represent only a small part of the overall MHC content. Furthermore tumor cells and surrounding stroma cells showed a completely distinct pattern of HLA presented peptides. With this approach we have identified new tumor specific MHC ligands derived from already established cancer related antiges (e.g. TMPRSS3, CA-125, hTERT) and could also reevaluate previously found peptides that were formerly claimed to be tumor specific and are now more likely to originate from the tumor stroma.
Conclusions: These results emphasize the importance of using specific cell subsets instead of bulk tissue for the identification of new tumor specific HLA ligands.

P1731

IL-2 expression enhances the anti-tumor effects of a DNA vaccine encoding HPV-16 E7 oncoprotein genetically fused with the HSV-1 gD protein

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Purpose/Objective: Cervical cancer is the second most lethal cancer among women and all cases are associated with high-risk Human papillomavirus (HPV) persistent infections. Cellular malignization is attributed to E6 and E7 HPV oncoproteins, which represent ideal targets to the development of immunotherapeutic strategies against HPV-induced tumors. In this regard, our group has developed a cervical cancer DNA therapeutic vaccine encoding HPV-16 E7 protein genetically fused to the glycoprotein D (gD) of herpes simplex virus type 1 (HSV-1). Previous results showed that the vaccine encoding

HPV-16 E7 fused to gD (pgDE7), when administered in a four dose vaccine regimen (100 μg of DNA/dose) intramuscularly, induced significant activation of E7-specific $CD8^+$ T cells and 40% of therapeutic anti-tumor effect in mice.

Materials and methods: As an attempt to enhance the observed therapeutic anti-tumor effects, we tested the co-administration of plasmid encoding IL-2 (pIL-2) to the DNA vaccine administered in saline solution by the intramuscular route. Immune responses and tumor protection monitoring were evaluated in immunized mice previously challenged with tumor cells expressing HPV-16 E6 and E7 proteins (TC-1 cells).

Results: Combination of plasmid pIL-2 with pgDE7 increased the therapeutic protection to 100% after a single dose administration using only 50 μg of each plasmid. Co-administration of plasmid encoding IL-2 to pgDE7 DNA vaccine also increased the $CD8^+$ T cells IFN-g production and cytotoxic potency, as well as total and E7-specific $CD8^+$ T cell tumor infiltration when TC-1 cells were inoculated suspended in Matrigel. Moreover, IL-2 concomitant expression with the protein encoded by the DNA vaccine did not enhance the activation of regulatory T cells or myeloid derived suppressor cells in tumor bearing mice.

Conclusions: In summary, the data presented in this study describes the development of a new and potent therapeutic vaccine against HPV-16-associated tumors and encourages its further evaluation in clinical trials.

P1733

Immunogenic MUC1 glycopeptides can be generated when dendritic cells (DCs) are primed with microvescicle bound MUC1 tumor associated glycoprotein, but not with soluble MUC1

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Purpose/Objective: Efficient antigen cross-processing by Dendritic Cells (DCs) is a requirement to obtain efficacious anti-tumor immune responses (IR). Block of endocytated tumor associated antigen (TAA) in the early compartments of the intracellular processing machinery shifts the IR towards a Th2 balance. MUC1 is one of the most relevant tumor associated glycoprotein expressed by epithelial cells. Its soluble form has shown to be blocked in MHCII compartment of DCs. On the other hand, tumor associated glycoforms of MUC1 have been shown to be immunogenic *in vivo*.

Objective of this study is to investigate the processing pathways of different MUC1 formulations in DCs and to characterize the repertoire of glycoepitopes thus generated.

Materials and methods: MUC1 carrying microvesicles (MVs) were purified from ascites or from supernatant of MUC1 transfected DG75 cells. CHO-K1 and CHO-*ldl* cells were used to produce recombinant MUC1 carrying ST and Tn carbohydrates (ST-MUC1 and Tn-MUC1 respectively). Confocal microscopy and WB were used to study MUC1 intracellular localization in DCs after uptake. FACS and ELISA were employed to characterise MUC1 glycoforms with specific MoAbs.

Results: MUC1 as soluble molecule, independently by the glycosylation profile, appears to be blocked in the pre-endosomal compartment. Receptor-mediated endocytosis pushes further the processing in the HLAII compartment while only MUC1 carried by MVs localizes in both HLAII and HLAI compartments in DCs. The distinct processing of the MUC1 membrane bound is accompanied also by deglycosylation processes generating Tn-MUC1 immunogenic glycoepitopes as identified by the use of glycoepitope specific MoAbs.

Conclusions: These results strongly suggest that the antigen formulation is of crucial importance both for cross-processing and both for the generation of immunogenic glycoepitopes. Moreover the glycosylation complexity of the immunogen may be an additional parameter to be considered in the design of DC-based cancer vaccines.

P1735

Improved anti-tumor cellular response by combined therapy in an experimental model

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Purpose/Objective: Neither radiotherapy alone nor injection of dentritic cells (DCs) can successfully control the tumor growth. Radiation induces tumor cell apoptosis, resulting in the release of tumor antigens and danger signals, which are favorable for DC capturing antigens. Hence, the strategy of combined irradiation and activated DC vaccine may be a novel approach for inducing cellular anti-tumor response.

Materials and methods: WEHI-164 a Balb/c derived fibrosarcoma cell line was injected subcutaneously to female Balb/c mice. Bone marrow cells were cultured with GM-CSF and IL-4 for 5 days. Listeria monocytogenes antigens and tumor cell lysate had been added for another 2 days. Seven days after tumor challenge, localized single does (10 Gy) irradiation was applied. Activated DCs were injected SC around the tumor, the day after irradiation.

Tumor size was monitored every other day. Two weeks after immunization the splenocytes were isolated and cytotoxic activity was measured with a lactate dehydrogenase cytotoxicity detection kit. Intercellular INF_γ assessment was performed using a FACScalibur cytometer with WinMDI2.9 software.

Results: It has been shown immunotherapy with DCs after irradiation has more potent cellular response in comparison with irradiation alone, especially using activated Listeria monocytogenes DCs. The cytotoxicity of splenocytes in combined therapy specially in the group which received complete cocktail was more than other groups ($P < 0.016$).

Evaluation of intra-splenocyte INF_γ staining showed significant increase in the group of animal which had received both local irradiation and activated DC injection ($P < 0.001$).

Monitoring tumor size and mice survival showed reduced tumor size and increased survival in the combination treatment group ($P = 0.011$, $P < 0.001$).

Conclusions: Combination therapy using activated Listeria monocytogenes DCs and local irradiation resulted to achieve a more desirable and efficient cellular anti-tumor response against poorly immunogenic tumor as well as increased mice survival with tumor regression.

Therefore co-administration of local irradiation and activated DCs may be a promising strategy for inducing a potent response to radio sensitive solid tumors.

P1736

Improving dendritic cell vaccine immunogeneicity by lipid nanoparticle-mediated delivery of PD-1 ligand siRNA combined with target antigen mRNA electroporation

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Purpose/Objective: Dendritic cell (DC)-based vaccination boosting antigen-specific immunity is being explored for the treatment of cancer and chronic viral infections. Although DC-based immunotherapy can induce immunological responses, its clinical benefit has been limited, indicating that further improvement of DC vaccine potency is essential.

Materials and methods: In this study, we explored the generation of a clinical-grade applicable DC vaccine with improved immunogenic potential by combining PD-1 ligand siRNA and target antigen mRNA delivery.

Results: We demonstrated that PD-L1 and PD-L2 siRNA delivery using DLin-KC2-DMA-containing lipid nanoparticles (LNP) mediated efficient and specific knockdown of PD-L expression on human monocyte-derived DC. The established siRNA-LNP transfection method did not affect DC phenotype or migratory capacity, and resulted in acceptable DC viability. Furthermore, we showed that siRNA-LNP transfection can be successfully combined with both target antigen peptide loading, as well as mRNA electroporation. Finally, we demonstrated that these PD-L silenced DC loaded with antigen mRNA superiorly boost *ex vivo* antigen-specific $CD8^+$ T cell responses from transplanted cancer patients.

Conclusions: Together, these findings indicate that our PD-L siRNA-LNP modified DC are attractive cells for clinical-grade production and *in vivo* application to induce and boost immune responses not only in transplanted cancer patients, but likely also in other settings.

P1737

IVAC: individualized vaccines for cancer

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Purpose/Objective: Cancer is driven by multiple genetic events followed by further clonal evolution, rendering disease elimination with single-targeted drugs a difficult task. The multiplicity of gene mutations derived from sub-clone heterogeneity may represent an ideal setting for multi-epitope tumor vaccination. Vaccines are particularly suited for the activation of antigen-specific $CD4^+$ and $CD8^+$ T cells and allow precise targeting of tumor-associated molecular alterations. In the last years, multiple tumor- associated antigens (TAA) have been identified and categorized based on tumor-specific deregulation of gene expression. In addition, neo-epitopes can be created by non-synonymous, somatic mutations. As human cancers carry 100–300 non-synonymous mutations on average which are not subject to central immune tolerance, these mutations can be ideal candidates for individual vaccine development. We propose that cancer can be targeted by T cells induced by poly-neo-epitopic vaccines based on non-synonymous individual tumor-specific mutations.

Materials and methods: In order to test this hypothesis, we resorted to B16-F10 murine melanoma of which we have identified more than 500 non-synonymous mutations by whole exome sequencing. After selection of highly expressed genes and potential MHC binding affinities of the respective mutated epitopes, 50 mutations were chosen and validated by Sanger sequencing. In order to define the immunogenicity of the mutation-coding sequences, we designed 27-meric peptides incorporating either the mutated or the wild-type amino acid to immunize C57BL/6 mice and determined the immune response by *ex vivo* IFNg ELISpot.

Results: We found one third (16/50) of the mutation-coding peptides to be immunogenic, and out of these, 60% elicited immune responses preferentially directed against the mutated sequence as compared to the wild-type sequence. Anti-tumor potency of these epitopes was confirmed in a transplantable B16-F10 melanoma model where mice immunized with mutation-encoding peptides revealed tumor control in the protective and the therapeutic setting.

Conclusions: Thus we successfully showed that mutated epitopes containing single amino acid substitutions qualify as effective vaccines. Our study provides a comprehensive picture of the B16-F10 mutanome and strongly suggests that the combination of deep sequencing with systematic immunogenicity analysis of non-synonymous point mutations paves a new path for the individualized immunotherapy of cancer patients.

P1738

Low affinity HLA-A2 restricted melanocortin 1 receptor-derived peptides are recognized by melanoma-specific CTLs and TILs from melanoma patients

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Purpose/Objective: Melanocortin 1 Receptor (MC1R) is expressed on a majority of fresh melanoma biopsies and cell lines. Three low-affinity HLA-A2-restricted highly hydrophobic MC1R-derived peptides [MC1R291 (AIIDPLIYA), MC1R244 (TILLGIFFL) and MC1R283 (FLALIICNA)] can elicit cytotoxic T-lymphocytes (CTL) responses from normal donor peripheral blood lymphocytes (PBL). Peptide-specific CTL recognized MC1R-transfected targets as well as a panel of MHC class I-matched melanomas, demonstrating that MC1R epitopes are naturally processed and presented by human melanoma cell lines. Here, the presence of anti-MC1R specific CTLs precursors was assessed in a population of melanoma-specific T cells derived from PBL and tumour-infiltrating lymphocytes (TIL) from HLA-$A2^+$ melanoma patients.

Materials and methods: Tumour tissues and/or blood samples from eleven advanced stage IV melanoma patients were obtained and TILs, melanoma-specific CTLs and melanoma cell lines were generated from these samples. Cytotoxic activity was evaluated using a standard Cr^{51} release assay and MC1R-specific populations were obtained by tetramer staining and limiting dilution.

Results: Anti-MC1R HLA-A2-restricted cytotoxic activity was detected in 50% of TILs derived from HLA-$A2^+$ melanoma patients. While 5/10 CTLs lines recognized either MC1R244 or MC1R291, 2/10 CTLs lines also recognized HLA-A2/MC1R283 complexes. 4/5 HLA-A2/MC1R-specific CTLs lines displayed also cytotoxic activity against three HLA-A2-restricted gp100-derived epitopes ($gp100_{280-288}$; $gp100_{457-466}$ and $gp100_{476-485}$) demonstrating TILs multiple epitope-specificity. Moreover, two HLA-A2/MC1R-specific CTLs lines cross-reacted with the immunodominant MART-1/Melan-A_{27-35} peptide. Furthermore, 3/5 MART-1-specific TILs recognized only HLA-A2/MART-1_{27-35} complexes. Additionally, it should be noted

that the existence of MC1R244-reactive CTLs was demonstrated at a clonal level. Finally, using tetramers containing MC1R-derived peptides, anti-MC1R T cell subsets were detected in TILs populations from melanoma patients.

Conclusions: Taken together, our data support that HLA-A2-restricted MC1R-derived peptides are common immunogenic epitopes for melanoma-specific CTLs and may thus be useful for the development of melanoma peptide-based immunotherapy.

P1741

Monitoring of ADCC with a Fc-optimized CD19 antibody in post-transplant MRD treatment of paediatric patients with refractory B-lineage acute lymphoblastic leukemia

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Purpose/Objective: Allogeneic stem cell transplantation is one of the major therapeutic options in paediatric patients with refractory B-lineage acute lymphoblastic leukemia. Despite significant improvement of bone marrow and peripheral blood stem cell (PBSC) transplantation over the last decade, relapse is still the major cause of death post-transplant due to persistent minimal residual disease (MRD). In order to prevent relapse in high-risk patients post-transplant, immunotherapy using a third-generation CD19 monoclonal antibody (mAb) is a promising approach and is deployed in an ongoing compassionate use program at our institution. Through its improved capability to recruit FcγRIIIA bearing effector cells, this Fc-optimized chimerized CD19 antibody mediates enhanced antibody-dependent cellular cytotoxicity (ADCC) by NK cells. Here we present preliminary results regarding ADCC efficacy and pharmacokinetics.

Materials and methods: Five paediatric patients with refractory B-lineage ALL received CD19 mAb (4G7SDIE) after allogenic stem cell transplantation. Donor derived PBMCs of the patients were analyzed by a 2-h europium release cytotoxicity assay. ADCC was measured by adding autologous serum taken after 4G7SDIE infusion or by adding 1 μg/ml 4G7SDIE to cryopreserved primary B-lineage ALL blasts *in vitro*. Titers of biologically active 4G7SDIE in patient serum samples were analyzed by a flow cytometry-based method. MRD levels were dermined by PCR and flow cytometry.

Results: Donor derived NK cells of the patients exerted insufficient lysis of primary B-lineage ALL blasts (mean specific lysis: 4.00%). However, lysis could be significantly increased by adding autologous patient serum taken after antibody treatment (mean specific lysis: 20.33%, $P = 0.0001$, student's t-test) or by adding 1 μg/ml 4G7SDIE (mean specific lysis: 34.84%, $P < 0.0001$). Activity of patient NK cells was similar to NK cells of healthy donors. ADCC relevant serum titers of biologically active 4G7SDIE were detectable up to 7 days after antibody infusion. In 3/4 patients significant MRD reduction was achieved.

Conclusions: ADCC mediated antileukemic effects for the 4G7SDIE antibody treatment have been shown *in vitro*. A further clinical trial will be necessary to investigate potential effects of this therapeutic antibody *in vivo*.

P1742

Mucosal imprinting of vaccine induced-CD8+ T cells is crucial to inhibit mucosal tumors

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Purpose/Objective: Although many human cancers are located in mucosal sites, most [F1] cancer vaccines were [F2] tested against subcutaneous tumors in preclinical models. We therefore wondered whether mucosa-specific homing instructions to the immune system might influence mucosal tumor outgrowth. We showed that the growth of orthotopic head and neck or lung cancers was only inhibited when a cancer vaccine was delivered by the intranasal (i.n) mucosal and not the intramuscular (i.m) route.

Materials and methods: We set up original orthotopic models of head and neck and lung cancers (on B6 mice injected with TC1 cells expressing the HPV16 E7 protein) and a cancer vaccine strategy based on a non replicative delivery system, the B subunit of Shiga toxin as mucosal vector which has previously been shown to target antigen to dendritic cells.

We analyzed the expression of chemokine receptors and integrin molecules expressed by T cells after intranasal or intramuscular immunization with STxB-E7 at mucosal and systemic compartments.

We blocked CD49a interaction with anti-CD49a mABs to demonstrate his role on T cell trafficking to orthotopic tumors.

Results: We found that mucosal immunization induced a response compartmentalized to the regional mucosal site for the $CD8^+$ T cells response, as we did not detect antigen specific $CD8^+$ T cells in genital draining lymph node after i.n immunization. Also we demonstrated a preferential recruitment of $CD8^+$ T cells in mucosal head and neck tumors after intranasal immunization which may be explained by the role of DC at the site of initial priming to imprint homing receptor expression onto T cells favouring their homing to the site of their initial activation. We found that intranasal immunization preferentially upregulated CD49a and CD103 on $CD8^+$ T cells at mucosal site. Intratumoral E7-specific T cells expressed high levels of CD49a, the $CD8^+$ T cells infiltrating head and neck cancer after i.n STxB-E7 administration significantly decreased when the vaccine was co-administered with anti-CD49a mAb and partially inhibited the therapeutic efficacy of the i.n STxB-E7 vaccine.

Conclusions: We provide direct evidence for the compartmentalization of mucosal tumor immunity, a critical finding for the rational design of better cancer vaccines. This work demonstrates for the first time that the efficacy of a mucosal cancer vaccine for head and neck or lung cancer is linked to its ability to trigger a mucosal program in vaccine-elicited $CD8^+$ T cells, which may represent a prerequisite and a surrogate marker of clinical efficacy.

P1743

Naive T cells showing HLA-restricted reactivity against unpulsed autologous dendritic cells hamper enrichment by *in vitro* priming of low-frequency antigen-specific T cells

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Purpose/Objective: *In vitro* induction of primary T cell responses by priming of naïve T cells against selected antigens (Ags) is feasible, but still poorly reproducible. In recent experiments we observed a profound proliferative response of human adult or cord blood-derived naïve T cells against autologous dendritic cells (autoDCs) in the absence of foreign Ags. This autoDC reactivity hampered the enrichment of low-frequency Ag-specific T cells.

Materials and methods: Purified, PKH-labeled naïve T cells (CD3+CD45RO-CD27+) were exposed to unpulsed autoDCs (R/S ratio 1/1) for 7–10 days in medium supplemented with 10 ng/ml IL-7 and 0.1 ng/ml IL-15. Based on their proliferative response, autoDC-reactive (PKH-low) and non-reactive (PKH-high) T cells were isolated 1 cell/well or in bulk by flow cytometric cell sorting. Secondary stimulations were done with autologous or allogeneic HLA-(mis) matched stimulator cells with or without HLA-blocking antibodies.
Results: AutoDC reactivity was found to be mediated by a polyclonal CD4+ and CD8+ T cell response, which occurred even under entirely serum-free conditions and could be inhibited by Tregs. The autoDC-reactive T cells, representing 1–3% of the total naïve T cell pool, acquired CD45RO expression, while the non-reactive counterparts remained naïve in response to autoDCs, but could still mount Ag-specific responses. Although both CD4+ and CD8+ naïve T cells showed autoDC reactivity, we could only generate CD4+ clones retaining their reactivity against autoDCs. The autoDC-reactive CD4+ clones showed IFN-γ and/or IL-4 production, and TCR/CD3 down-regulation upon stimulation, but most of them were not cytotoxic against autoDCs. Moreover, these clones responded to autologous monocytes, and immature DCs, but not to non-myeloid cells (EBV-LCL, PHA-blasts and (activated) B cells), indicating their myeloid-lineage specific reactivity. A panel study with allogeneic DCs matched for 1 or more of the HLA class II molecules and analyses using HLA blocking antibodies revealed that from individual donors both HLA-DP, -DQ and -DR restricted clones were isolated, further illustrating the polyclonality of this autoDC reactivity.
Conclusions: Our data showed the existence of T cells in the normal naïve repertoire harboring HLA-restricted and myeloid lineage-specific reactivity against autologous and HLA-matched allogeneic cells.

P1744
Nanobodies as potential diagnostic tools for *in vivo* imaging of lymphomas

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Purpose/Objective: There is a great potential for probes and techniques that allow for a specific detection of tumors *in vivo*. While monoclonal antibody-based probes offer good target specificity there has been a move towards smaller probes, which circumvent obstacles presented by conventional antibodies. Here we compared llama-derived nanobodies as alternative to monoclonal antibodies analysing the *in vivo* fluorescence imaging properties of lymphomas in a mouse model.
Materials and methods: In our study we used lymphoma cells that were transfected with the ecto-enzyme ART2 and compared the ART2-specific monovalent nanobody *s+16a* (15 kD) with a conventional monoclonal rat IgG_2a antibody (*Nika102*, 150 kD). Both constructs were labeled with the near-infrared fluorochrome AlexaFluor680. Athymic nude mice, bearing both, ART2-positive and ART2-negative lymphomas, were injected i.v. with the AF680-conjugates and monitored over 24 h. Properties of AF680-conjugates were investigated *in vivo* and *ex vivo* using near-infrared fluorescence (NIRF) imaging. Individual differences in tissue penetration and target binding efficiency were further analyzed by flow cytometry and fluorescence microscopy of dissected tumors.
Results: *In vivo* imaging revealed significant differences between the two antibody constructs in terms of biokinetics, biodistribution and labeling efficiency, which could be confirmed by *ex vivo* flow cytometry and fluorescence microscopy of dissected tumors. Both constructs revealed specific labeling of ART2-positive tumors but not of ART2-negative tumors. Rapid renal elimination of *s+16a* resulted in a good signal-to-background ratio of ART2-positive tumors *in vivo* already 6 h after injection. By contrast, the larger construct *Nika102* showed longer persistance in the plasma, leading to diffuse background signals with tumor-to-normal tissue ratios significantly lower as compared to nanobody *s+16a*..
Conclusions: Our results highlight the advantages of nanobody-conjugates as imaging tools for the specific detection and characterization of lymphomas *in vivo*. Compared to larger conventional antibodies, nanobodies are characterized by their favorable biodistribution and high signal-to-background ratio, which makes them particularly suited for short-term diagnostic imaging.

P1745
Naturally presented HLA ligands derived from histone deacetylases provide novel targets for T-cell mediated immunotherapy of ovarian carcinoma

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Purpose/Objective: Late diagnosis and resistance to chemotherapy are the main reasons for the high mortality among women suffering from ovarian carcinoma (OvCa). New strategies to circumvent this drug resistance are urgently needed. One approach is to develop immunotherapies, including peptide-based cancer vaccines which should induce tumor-specific T cells. This strategy requires the identification of MHC-presented, immunogenic peptides derived from tumour-associated antigens (TAA).

The purpose of this study is to identify novel peptides for developing a multipeptide vaccine targeting OvCa.
Materials and methods: MHC class I ligands of OvCa samples were analysed by liquid chromatography coupled mass-spectrometry. Peptides from TAA or proteins which might be involved in tumorigenesis were selected for *in vitro* priming performed with PBMCs from healthy blood donors. T cells were isolated by magnetic activated cell sorting and stimulated with peptide-pulsed autologous DCs and B cells.
Results: Altogether we identified 8544 HLA ligands from nine different OvCa samples including 25 peptides derived from TAA. Up to know, *in vitro* priming of these candidate peptides revealed eight immunogenic epitopes.

The most promising epitope we found is an HLA-A*02 restricted peptide derived from HDAC1 (histone deacetylase 1), an established TAA highly overexpressed in OvCa and associated with poor prognosis. *In vitro* priming of healthy PBMCs induced T-cell responses directed against the HDAC1 peptide in 11 of 21 donors, as determined by positive intracellular stainings for IFNγ, TNFα, IL-2, CD107a and MIP-1β. In addition, those T cells were cytotoxic shown by lysis of HLA-A*02$^+$ tumour cells in chromium release assays whereas HLA-A*02-negative cells were not killed.

Moreover this peptide is also a natural HLA-A*02 ligand of HDAC2 and has been identfied on several other tumor types, allowing the simultaneous targeting of two different tumor-associated HDACs and the usage in vaccination trials for different tumor entities. Up to now 3 more HDAC1 dervied natural MHC peptides have been discovered suggesting a frequent presentation of HDAC1 peptides on tumor cells.
Conclusions: Based on our experimental data we are going to use the HDAC1/2 derived peptide in an upcoming multipeptide Phase I/II vaccination trial in HLA-A*02$^+$ OvCa patients.

P1746

Neutralization of IL-10 And TGFß1 rescues T cells from tolerogenic mechanisms induced by HER2 vaccine in cancer patients

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Purpose/Objective: Dendritic cells (DCs) are the most potent antigen presenting cells in the immune system, which are capable of priming naïve T cells and can stimulate memory T cells and B cells to generate antigen-specific responses. Recent clinical trials were designed to stimulate immune system against HER2+ tumor by using HER2-peptides-based or peptides-loaded-DCs vaccine, but no clinical reponses was elicit, probably due to self tolerance mediated by natural and peripheral-induced Tregs.
Materials and methods: In our work, CD14+ monocytes were separated from PBMC of healthy subjects or patients with pancreatic and breast cancer overexpressing HER2, to generate dendritic cells (DCs). This DCs were matured and transfected with plasmids coding for extracellular and transmembrane domains of human HER2 then used to activate autologous T cells. Activation of T cells were assessed by intracellular staining, IFNγ-ELISpot, ELISA and cytotoxicity assays.
Results: Results showed that Human-DCs were able to trigger IFNγ release by autologous T cells in healthy donors, but not in cancer patients. Failure of Human-DCs could be ascribe to higher induction of Tregs activity and to higher amount of IL-10 and TGFβ1 found in cultures, since neutralization of these two cytokines restored the ability to induce IFNγ production.
Conclusions: From these we can conclude that cancer vaccine based on self antigen can induce an antitumor response but also activate immune tolerance mechanisms. This finding represent an important point thatbe consider to design cancer vaccines in future.

P1747

Next generation HLA ligandomics: defining the targetome for the immunotherapy of cancer

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Purpose/Objective: The identification and selection of appropriate targets is basic prerequisite for the success of peptide based immunotherapy of cancer. Due to the complex mechanisms underlying antigen presentation, indirect approaches towards target identification (e.g. reverse immunology) cannot warrant success. Furthermore the patient specific individuality of malignancies calls for flexible and informed selection of targets (from a vaccine warehouse) rather than standardized vaccine cocktails. Thus the objective was to devise a strategy to characterize the malignancy derived HLA ligandome in a direct, semi-quantitative and patient individualized manner.
Materials and methods: HLA ligands were isolated from bulk tumor tissue and corresponding autologous benign tissue of clear cell renal cell carcinoma (ccRCC) patients. Liquid chromatography coupled mass spectrometry (LC-MS/MS) based peptide sequencing in technical replicates and subsequent reproducibility analysis yielded semi-quantitative information of ligand and source protein representation in malignancy and benign tissue derived HLA ligandomes.
Results: Exhaustive HLA ligandome analysis was performed for several patients yielding more than 2500 HLA ligand identifications in every instance. KEGG pathway analysis and literature research showed great concordance of high-ranking tumor associated peptides (TUMAPs) and tumor associated antigens (TAAs) identified by our approach with antigens implicated in ccRCC and cancer in general (e.g. WT1, MUC1, EGFR, JUNB, MYC, NNMT). Furthermore we identified a large array of novel TAAs and corresponding HLA ligands. A high grade of tumor individuality became evident from overlap analysis of the top ranking TAAs from all samples analyzed.
Conclusions: The presented strategy enables the identification of novel TAAs directly based on the immunologically pivotal feature of HLA restricted presentation while readily providing the corresponding HLA ligands. Proof of principle was provided by the robust identification of well-known TAAs from all ccRCC samples analyzed. Furthermore this approach provides the rationale for the patient specific selection of the most promising peptide vaccine combination.

P1748

Oncogene-targeting T cells reject large tumors while oncogene inactivation selects escape variants in mouse models of cancer

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Purpose/Objective: Cancer cells are genetically unstable and, therefore, single therapies like chemotherapy or oncogene inactivating drugs frequently select resistant clones. A priori, one would expect that adoptive T cell therapy similarly selects escape variants.
Materials and methods: We established mouse cancer models, which allowed targeting of an oncogene by drug-mediated inactivation or monospecific $CD8^+$ effector T (T_E) cells. Cancer cells expressed a doxycycline (dox)-regulatable SV40 large T-firefly luciferase (TagLuc) fusion protein, which allowed oncogene regulation and visualization *in vivo*.
Results: Switching off TagLuc expression in large tumors resulted in rapid regression, followed by growth of dox-unresponsive variants. Each variant revealed a unique point mutation in the dox-binding domain of the transactivator, demonstrating a seemingly unlimited reservoir of genetic variants in the tumor. In contrast, Tag peptide I-specific T_E cells completely rejected large tumors ($\geq$500 mm^3), which did not require antigen cross-presentation by stromal cells. While drug-mediated oncogene inactivation selectively killed the cancer cells and left the tumor vasculature intact, which likely facilitated survival and growth of resistant clones, T_E cell treatment led to blood vessel destruction and probably 'bystander' elimination of escape variants.
Conclusions: Adoptive T cell therapy and drug-based cancer treatment were both highly effective but only T cells killed cancer cells and simultaneously destroyed the tumor vasculature, which may be critical to prevent escape.

P1749

Overcoming immunoescape mechanisms and induction of CD8$^+$ T cell-mediated resistance to tumor by polymer-bound doxorubicin conjugate targeted to tumor-specific antigen

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Purpose/Objective: This study was focused on interactions of immune system of the host with the tumor during and after the treatment course with doxorubicin bound to HPMA copolymer carrier and targeted with mAb to tumor-specific antigen.

Materials and methods: BALB/c mice bearing syngeneic BCL1 leukemia, a mouse model of human chronic lymphocytic leukemia, were used in this study. B1 mAb recognizing the idiotype of membrane IgM expressed on BCL1 cells was used to target HPMA copolymer with covalently bound doxorubicin to tumor cells.

Results: Treatment with the conjugate can cure up to 100% of mice and significant proportion of cured mice show long-lasting resistance to BCL1 leukemia. We show that both CD4$^+$ and CD8$^+$ T cells are required for establishment of the resistance, but only CD8$^+$ T cells are necessary for its maintenance. We found that BCL1 cells paradoxically express MHC I, II and also costimulatory molecules CD80 and CD86 which can aid eliciting of anti-tumor response. On the other hand, we figured out that BCL1 cells also employ several immunoescape mechanisms, such as expression of PD-L1, PD-L2 and IL-10. Moreover, BCL1 leukemia progression is accompanied by significant increase of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T (T_{reg}) cells relatively to other CD4 T$^+$ cells. In this study, we provide first direct evidence that expanded T_{reg} cells can indeed promote tumor progression by using mice with selectively expanded T_{reg} cells prior to inoculation of BCL1 leukemia. Finally, we have also shown that elimination of some immunoescape mechanism, e.g. deletion of T_{reg}, can significantly improve the therapeutic outcome of chemotherapy.

Conclusions: BCL1 cells can be recognized by BCL1-specific T cells, but instead of effective priming, such T cells are anergized or deleted by apoptosis. Treatment with doxorubicin bound to HPMA copolymer carrier and targeted specifically to the tumor cells is capable of overcoming these immunoescape mechanisms of BCL1 leukemia and establish long-lasting CD8$^+$ T cell-mediated tumor-specific resistance.

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P1750

PDE5 inhibitors for the immune therapy of head and neck squamous cell carcinoma

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Purpose/Objective: Head and neck squamous cell carcinoma (HNSCC) is a deadly disease with significant social and economic impact that is characterized by a strong accumulation of suppressive Myeloid Derived Suppressor Cells (MDSC) and macrophage and Treg in the perypheral blood and at the tumor site. We have previously shown in tumor murine models that PDE5 inhibitors (Tadalafil, Cialis, and Sildenafil, Viagra), commonly used for the treatment of erectile disfunction, can modulate the immune system by blocking MDSCs suppressive mechanisms and by promoting a spontaneous anti-tumor immunity. To verify if these findings hold true also in human, we are conducting a clinical Trial in which patients with HNSCC treated daily with tadalafil are immune monitored.

Materials and methods: Enrolled patients with Squamous cells carcinoma of the oral cavity, are treated with two doses of tadalafil or with placebo for 20 days before surgery. Immunological endpoints are evaluated before treatment, at the time of the surgery and 6 weeks after surgery. Functional assays, flow cytometry based characterization and immune fluorescence are performed on the blood and on the tumor specimen.

Results: Interim analyses indicate that MDSC and Treg are downregulated by PDE5 blockade in the peripheral blood and in the tumor and, more importantly, tumor specific immunity seems to be enhanced. Indeed, T cell proliferation stimulated by autologous dendritic cells pulsed with the autologous tumors is significantly increased after the *in vivo* regimen with Tadalafil. Furthermore, an higher infiltration of activated (CD69$^+$) CD8$^+$ T cells is observed by Immune fluorescence in the tumor specimen after tadalafil treatment compared to the pre-treatment baseline.

Conclusions: If our results will be confirmed when all the patients will be enrolled, PDE5 blockade will emerge as an effective and safe strategy to restore the optimal tumor micro- and macro- environment for an effective immunotherapy of HNSCC.

P1751

Peritumoral treatments with poly IC or MSU+ *M. smegmatis* enhance the anti-tumour response by activating both innate and adaptive immunity

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Purpose/Objective: Tumours can escape immune responses by subverting immune cells to an immuno-suppressive phenotype. We wished to investigate the potential of microbial stimuli and sterile danger signals, which are known to provide activation signals to dendritic cells (DCs) and other innate cells, to overcome this suppression and stimulate an effective anti-tumour immune response.

Materials and methods: We injected non-pathogenic *Mycobacterium smegmatis*, the TLR ligands CpG, poly IC and LPS, and the sterile danger signal monosodium-urate crystals (MSU) peri-tumorally into immuno-competent mice bearing established subcutaneous B16F1, B16-OVA or E.G7-OVA tumours. Tumour growth was recorded and changes in immune cell types, activation state and cytokine production were assessed by flow cytometry, multiplex assay and RT-PCR. Metastasis formation was studied in the orthotopic 4T1 breast carcinoma model. RAG1-/- mice and *in vivo* cell depletion were employed to assess the requirement of T cells and NK cells.

Results: We found that CpG and poly IC treatment delayed tumour growth, whereas LPS, *M. smegmatis* or MSU alone showed no effect. Combination of *M. smegmatis* with MSU, however, significantly delayed tumour growth and prolonged survival. In addition, treatment with poly IC or MSU+ *M. smegmatis* reduced the formation of metastases.

The anti-tumour immune response elicited by poly IC or MSU+ *M. smegmatis* treatment required CD8 T cells as well as NK cells. In addition, both treatments increased infiltration of CD8 T cells and NK cells in tumours and enhanced their capacity to produce IFN-γ and TNFα. Successful treatments induced expression of IL-6, iNOS, G-CSF and CXCL10 in the tumour, and correlated with an early increase in

inflammatory monocytes and as well as induction of monocyte-derived inflammatory DCs in the tumour-draining lymph nodes.
Conclusions: Poly IC and MSU+ *M. smegmatis* induce local inflammation that recruits inflammatory monocytes to the tumour site, and their maturation into DC. These cells may then take up tumour antigen and become sufficiently activated to migrate to the draining lymph node where they initiate the anti-tumour immune response. We conclude that selected adjuvants can modify the tumour environment to promote adaptive and innate immune responses and induce tumour rejection.

P1752

Pilot clinical trial of type 1 dendritic cells loaded with autologous tumor lysates combined with GM-CSF, pegylated IFN, and cyclophosphamide for metastatic cancer patients

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Purpose/Objective: Dendritic cell (DC) can be artificially manipulated to present tumor antigens either in the form of defined protein sequences or as antigenic material obtained from autologous tumor cells. We have tested the safety and biological activity of immunotherapy based on type 1 DC in advanced cancer patients. Our combination strategy was based on preclinical data that we developed in mouse models.
Materials and methods: Twenty-four patients with metastatic cancer received two cycles of four daily immunizations with monocyte-derived dendritic cells. DC were incubated with preheated autologous tumor lysate and subsequently with IFN-α, TNF-α, and polyinosinic: polycytidylic acid to attain type 1 maturation. One DC dose was delivered intranodally, under ultrasound control, and the rest intradermally in the opposite thigh. Cyclophosphamide (day 27), GM-CSF (days 1*4), and pegIFN alpha-2a (days 1 and 8) completed each treatment cycle.
Results: Pretreatment with cyclophosphamide decreased regulatory T cells to levels observed in healthy subjects both in terms of percentage and in absolute counts in peripheral blood. Treatment induced sustained elevations of IL-12 in serum that correlated with the output of IL-12p70 from cultured DC from each individual. NK activity in peripheral blood was increased and also correlated with the serum concentration of IL-12p70 in each patient. Circulating endothelial cells decreased in 17 of 18 patients, and circulating tumor cells markedly dropped in six of 19 cases. IFN-γ*ELISPOT responses to DC plus tumor lysate were observed in four of 11 evaluated cases. Tracing DC migration with [^{111}In] scintigraphy showed that intranodal injections reached deeper lymphatic chains in 61% of patients, whereas with intradermal injections a small fraction of injected DC was almost constantly shown to reach draining inguinal lymph nodes. Five patients experienced disease stabilization, but no objective responses were documented.
Conclusions: This combinatorial immunotherapy strategy is safe and feasible, and its immunobiological effects suggest potential activity in patients with minimal residual disease. A randomized trial exploring this hypothesis is currently ongoing.

P1753

Polyclonal stimulation of conventional T cells *in vitro* is sufficient to prevent acute graft versus host disease while maintaining anti-tumor activity

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Purpose/Objective: Acute graft versus host disease (aGvHD) is still a major complication of allogeneic bone marrow transplantations often precluding successful therapy of hematological malignancies. Alloreactive conventional, i.e. non-regulatory, T cells contained in the graft not only mediate the desired graft versus tumor (GvT) effect but they are also responsible for the induction of aGvHD. Therefore, novel immunotherapeutic approaches for aGvHD must aim at modulating T cell function without abrogating allo-reactivity.
Materials and methods: To this end we studied the aGvHD-inducing potential of conventional T cells from C57BL/6 mice polyclonally pre-expanded *in vitro* before transplantation into lethally irradiated allogeneic BALB/c recipients *in vivo*. As polyclonal T cell activators we used paramagnetic beads coated either with a superagonistic anti-CD28 mAb (CD28-SA) or with an anti-CD3 plus a conventional anti-CD28 mAb (co-stimulation).
Results: Both stimulatory protocols efficiently protected BALB/c recipient mice from aGvHD. Tracking experiments using carboxyfluorescein succinimidyl ester diacetate-labeled cells further revealed that *in vitro* pre-expansion did not abrogate allo-reactivity of the transferred T cells *in vivo*. Preserved allo-reactivity could also be observed in short-term *in vivo* killing assays against allogeneic target cells where CD28-SA-stimulated T cells showed superior cytotoxic activity in comparison to T cells pre-activated with co-stimulatory beads. Efficient killing in the short-term *in vivo* assays was predictive for long-term protection in the BCL-1 lymphoma model as CD28-SA-stimulated conventional T cells significantly increased the survival of lymphoma-bearing mice as compared to recipients receiving no allogeneic T cells.
Conclusions;Polyclonal stimulation of conventional T cells *in vitro*, thus, inhibited aGvHD induction *in vivo* while maintaining the desired GvT effect. This study was supported by a grant from the Wilhelm Sander-Stiftung (2005.133.2).

P1754

Potentiation of the production and cytotoxic activity of the FasL-based chimeric proteins by concomitant expression of the non-apoptotic FasL

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Purpose/Objective: One aim of the laboratory is to develop soluble FasL fusion proteins, an attractive approach to treat cancer by triggering Fas apoptosis. To induce a productive death signal, soluble form of FasL requires an oligomerization beyond the homotrimeric state. A polymeric soluble FasL chimera (pFasL) was thus generated by fusing to the soluble FasL an immunoglobulin-like domain. A second generation of chimeras was designed to selectively induce apoptosis of malignant cells by fusing targeting modules, such as gdTCR or HLA-A2, to the pFasL molecule. The purpose of this study consists into potentiating production and cytotoxic activity of these FasL-based chimeras. We hypothesized that concomitant expression of the non-apoptotic soluble FasL improves structure of the FasL-based chimeras.
Materials and methods: For this purpose, we co-transfected the cDNA encoding FasL-based chimeras and the sFasL into mammalian cells. Only, the FasL based-chimeric constructs contain a FLAG epitope

tag, in order to discriminate them from the sFasL molecule. The secreted amount of recombinant proteins was assessed by a specific ELISA and the chimeras cytotoxic activity performed with MTT or iodide propidium assays. Their biochemical structures were also analyzed by (FasL co-) immunoprecipitation and gel filtration experiments.
Results: The concomitant expression of the soluble FasL considerably increases the secretion of FasL based-chimeras. The increasing production fluctuates between two to ninefold depending on the complexity of the FasL-based unit constituting the polymers. We demonstrated that sFasL was indeed physically attached to the FasL-chimeras and remodels their polymeric structures with a decreased size. Interestingly, FasL incorporation drastically potentiated their cytotoxic activities without hindering cell targeting of the FasL-chimeras.
Conclusions: Taken together, we describe an original approach that allows the improvement of polymeric FasL-based chimeric proteins, both at the level of the cellular production, and at the level of the cytotoxic activity. This approach may apply to a broad variety of FasL-derived chimeras used for anti-tumor therapy.

P1755
Pre-clinical assessment of EpCAM as a target for antibody based therapies of gastric cancer

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Purpose/Objective: The epithelial cell adhesion molecule (EpCAM) is highly expressed in gastrointestinal malignancies. EpCAM is suggested to be a marker of cancer stem cells and to be involved in processes such as metastasis. These characteristics and its surface expression render EpCAM a highly interesting target for antibody-based immunotherapies. To this end new antibodies need to be identified and tested in appropriate preclinical models.
Materials and methods: We investigated two EpCAM-specific antibodies in a spontaneous model of gastric cancer, the CEA424-SV40-T antigen-transgenic mouse (SV40 TAg) both *in vitro* and *in vivo*. Antibodies were characterized by Biacore measurements. Expression of EpCAM was addressed by flow cytometry and immunofluorescence.
Results: Antibodies with murine or rat backbone had affinities for EpCAM in the nanomolar range and recognized recombinant and naturally processed murine EpCAM protein. Both the primary tumor and a cell line established from the SV40 TAg mouse expressed EpCAM at high levels (range 60−100% of all tumor cells). *In vitro*, both antibodies had cytotoxic activity through the classical pathway of complement activation (range 20−40% of cell lysis) and through antibody-mediated cell-dependent cytotoxicity (range 50−80% of cell lysis). When administered *in vivo*, the EpCAM-specific antibodies were found evenly distributed in the EpCAM-positive tumor as well as in other EpCAM-expressing tissues. Distribution of the antibodies tightly correlated with the expression of EpCAM in the given tissue. Next, tumor-bearing SV40 TAg mice were treated three times i. v. with 300 μg of anti-EpCAM antibody. Remarkably, survival in this otherwise therapy resistant model was prolonged on average by 11 days ($P = 0.06$).
Conclusions: EpCAM targeting antibodies demonstrate good cytotoxicity in an *in vitro* model of gastric cancer. First *in vivo* data indicate that our antibody specifically targets cancer cells in a spontaneous model of gastric cancer which is known to be therapy-resistant. These data are of high relevance, as *in vitro* cytotoxicity as well as penetrance and accumulation into a tumor are prerequisites for *in vivo* use. Our data suggest that EpCAM represents an attractive target for the treatment of gastric cancer

P1756
Predicting tumor infiltrating lymphocyte (TIL) adoptive transfer clinical response in metastatic melanoma patients by T cell subpopulation-based rules

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Purpose/Objective: Tumor-infiltrating lymphocytes (TILs) are an immune population composed of different lymphocytic subpopulations (mostly T cells), that are derived from a tumor mass and have specificity and potential reactivity against the tumor. TILs are used in a clinical protocol called adoptive cell transfer for metastatic melanoma treatment with a success rate of about 50%. The collective outcome of using such a heterogeneous cell population is dictated by a complex network of interactions that span the molecular, cellular, and environmental levels. In a Previous study, we showed that the in-vitro reactivity of the TILs can be predicted from their subpopulation composition.
Materials and methods: In this study, we used machine learning techniques for retrospective analysis of the clinical in-vivo response of TIL treatment of 55 melanoma patients, aiming to find rules connecting TIL subpopulation frequencies to the clinical response.
Results: We have found that a simple set of subpopulation-based rules can predict the clinical response with an accuracy of 85−95%.

P1757
Rapid analysis and selection of antigens with clinical relevance for T cell therapy of hematological malignancies by microarray gene expression analysis

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Purpose/Objective: Patients with hematological malignancies can be successfully treated with allogeneic hematopoietic stem cell transplantation (alloSCT). In HLA-matched alloSCT, donor T cells can mediate beneficial Graft versus Leukemia (GvL) reactivity due to recognition of minor histocompatibility antigens (MiHA) on patient malignant cells. These T cells may also induce Graft versus Host Disease (GvHD) when ubiquitously expressed MiHA are recognized. Novel MiHA are now efficiently identified by whole genome association analysis. In this study, we investigated whether antigens can be rapidly selected for clinical relevance for T cell therapy of hematological malignancies by microarray gene expression (MGE) analysis.
Materials and methods: Various (malignant) hematopoietic cells and non-hematopoietic cells from target tissues in GvHD were collected. Non-hematopoietic cell types were also cultured in the presence of IFN-γ to mimick the inflammatory milieu early after alloSCT. Total RNA was isolated and processed for MGE profiling. T cell recognition of malignant hematopoietic and (cytokine treated) non-hematopoietic cell types was measured by IFN-γ ELISA.
Results: Quality control of the MGE database included cell type specific markers and expression patterns of well-known tumor associated antigens. Expression profiles of 15 novel MiHA were investigated by MGE. The genes encoding these antigens were all broadly expressed, except for two hematopoietic restricted genes,

which included ARHGDIB and EBI3. EBI3 is selectively expressed in mature dendritic cells, whereas ARHGDIB is expressed in all hematopoietic lineages. T cell experiments showed that 11 of the 15 antigens were recognized on (cytokine treated) fibroblasts. The four remaining antigens included the two selected hematopoietic restricted as well as two broadly expressed antigens, which were not selected due to potential *in vivo* reactivity towards non-hematopoietic tissues by higher avidity T cells. The experiments confirmed recognition of the ARHGDIB antigen on primary leukemic cells, illustrating its therapeutic relevance.

Conclusions: Our data demonstrate that gene expression analysis allows rapid analysis and selection of clinically relevant antigens for novel T cell therapies aiming to induce a more favorable balance between GvL/GvHD after alloSCT.

P1758

Reactivated human endogenous retrovirus K (HERV-K) in cancer patients: new antigens for cancer vaccination

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Purpose/Objective: Around 8% of the human genome consists of human endogenous retrovirus (HERV) sequences and HERVs can be further subdivided into 20 different families. HERV-K is the only known family encoding all structural and enzymatic proteins and is able to form retroviral particles however these particles are not infectious. Enhanced expression of HERV-K genes accompanied by the emergence of anti-HERV-K antibodies has been observed in patients for different tumor entities (e.g. germ cell tumor; melanoma). Because HERV-K expression is usually silenced in non-malignant cells, it fulfils the requirements to be used as a tumor-associated antigen (TAA).

Materials and methods: We generated recombinant poxvirus vaccine candidate based on the highly attenuated, avirulent vaccinia virus MVA (modified vaccinia virus Ankara). We inserted the HERV-K Env gene into the MVA genome and confirmed transgene expression. Mice immunized with the recombinant MVA were protected from engraftment of HERV-K Env expressing syngenic tumor cells. In addition to this prophylactic setting, mice had significantly reduced metastasis count in a therapeutic vaccination approach. Protection of mice correlated with an increase of HERV-K Env-specific lymphocytes. In addition, Balb/C mice immunized with the recombinant poxviruses showed a humoral immune response against HERV-K Env protein.

Results: Vaccination of mice with a recombinat MVA expressing HERV-K-Env as a phophylactic or a therapeutic approach showed significant anti-tumor effects.

Conclusions: Our results indicate that the generated recombinant poxvirus is suitable to induce a strong HERV-K-directed immune response and thus might be promising vaccine candidate against tumor entities which show a reactivated expression of HERV-K.

P1760

Selective targeting of membrane Hsp70 positive tumour cells by recombinant human granzyme B: a novel therapeutic approach for cancer?

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Purpose/Objective: One way by which natural killer cells and cytotoxic T cells are able to induce apoptosis is via the delivery of serine proteases such as granzyme B into target cells. Although perforin has been traditionally thought to be necessary for granzyme B uptake, current opinion over the precise role of perforin in the uptake mechanism is now divided. Even in the absence of perforin, it is possible for granzyme B to enter a target cell and induce apoptosis if that cell expresses a membrane form of heat shock protein 70 (Hsp70). This membrane form of Hsp70 is expressed in a large proportion of tumours (~50%), but not healthy tissue. Granzyme B could therefore provide a novel strategy for targeting tumours.

Materials and methods: To better investigate the role of membrane Hsp70 and granzyme B in tumour targeting, a method for producing biologically active recombinant human granzyme B was developed. For this, human granzyme B was stably transfected into the human embryonal kidney cell line HEK293. The addition of a (His)6 tag ensured successful protein retrieval from the cell supernatant and an inactivation site protected the HEK293 cells from apoptosis. Granzyme B was purified using a nickel column and activated by enterokinase digestion of the inactivation site. The resulting protein exhibits high purity and enzymatic activity.

Results: Granzyme B induction of apoptosis in membrane Hsp70 positive tumour cell lines (MCF7, CT26, 4T1) was observed both by morphological changes and by caspase-3 activation. There was evidence of apoptosis occurring after 4 h, with complete cell death after 24 h. Flow cytometric and confocal microscopy analysis reveals that the binding and uptake of granzyme B requires glycosylation. Blocking studies demonstrated that neuraminic acid was the most important for efficient granzyme B uptake. Current work is examining the intracellular pathways of granzyme B uptake using confocal microscopy of cells that are co-stained with antibodies specific for endosomal (early with Rab-4 and -5, late with Rab-7 and -9, recycling with Rab-11, -15, -17 and -25) and lysosomal (LAMP1 and LAMP2) compartments, and MitoTracker Green dye to identify the mitochondria.

Conclusions: Taken together, these findings indicate that granzyme B could offer a new therapeutic option for the targeting cancer in those patients that bear membrane Hsp70 positive tumours.

P1761

Spontaneous $CD4^+$ T-cell responses against indoleamine 2,3-dioxygenase

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Purpose/Objective: Indoleamine 2,3-dioxygenase (IDO) suppresses T cell immunity in the tumor microenvironment and IDO expression is commonly elevated in tumors and draining lymph nodes. Recently, we described cytotoxic CD8+ T-cell reactivity against IDO. In the present study, we show that CD4+ helper T cells additionally spontaneously recognize IDO. Hence, we scrutinized the vicinity of the previously described HLA-A*0201-restricted IDO-epitope for CD4+ T-cell epitopes.

Materials and methods: We demonstrated the presence of naturally occurring HLA class II-restricted, IDO-specific CD4+ T cells in cancer patients and in healthy donors using INFγ, TNFα and IL-17 EliSpot assay.

Results: IDO-reactive CD4+ T cells released INF-γ, TNF-α, as well as IL-17. In some donors, we observed that activation of IDO-reactive CD4+ T cells suppressed IL-10 production, whereas in PMBC from additional donors we could detect IL-10 release in response to the class II-restricted IDO-derived peptide.

Conclusions: IDO-specific T cells may participate in immune-regulatory networks where the activation of pro-inflammatory IDO-specific CD4+ responses may well overcome or delay the immune suppressive actions of the IDO-protein, which are otherwise a consequence of the early expression of IDO in maturing antigen presenting cells. In

contrast, IDO-specific regulatory T cells may enhance IDO-mediated immune suppression.

P1763

The 5T4 tumour associated antigen as a target for CAR-T cell therapy

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Purpose/Objective: The 5T4 oncofetal antigen is expressed on many tumour types including renal, colorectal, ovarian and gastric carcinomas but has limited expression on normal adult tissues. Hence, 5T4 is an attractive target for immune-based cancer therapies. In this study, we develop and test mouse 5T4 (m5T4) specific Chimeric Antigen Receptor (CAR) T cells for *in vitro* functionality.

Materials and methods: A m5T4 specific CAR was generated by amplification of the relevant single chain antigen-binding domain (scFv) from a high-affinity m5T4 specific antibody-producing hybridoma cell line and fusing to the CD3z transmembrane and signalling domain. Retroviral vectors encoding this m5T4 specific CAR were used to transduce C57 bl/6 T cells.

Results: Over multiple experiments, T cells were efficiently transduced to a level of approximately 75%. These CAR-T cell populations showed antigen-specific cytotoxicity against mouse tumours that expressed the m5T4 antigen but only background levels of activity against tumour cell lines lacking the target antigen. Moreover, T cells bearing the m5T4 scFv fused to a non-signalling transmembrane anchor failed to lyse $5T4^{+}$ target cells indicating that target binding and signalling by the CAR was required for effector T cell function. m5T4 CAR-T cells also produced significant levels of IFNg after co-culture with antigen-expressing target cells.

Conclusions: These observations confirm that a CAR specific for the mouse 5T4 antigen is functional in mouse T cells and warrants further examination of anti-tumour efficacy in relevant *in vivo* model systems.

P1764

The anti-tumor effects of an adenoviral vaccine expressing 4-1BB ligand in a murine melanoma model

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Purpose/Objective: In therapeutic tumor vaccination, replication deficient adenovirus expressing a glycoprotein of LCMV linked to the MHC class II associated Invariant chain (Ad5-IiGP) was found to delay B16.F10 tumor growth expressing the epitope GP^{33-41}. However, in this mouse model, a complete regression of established palpable tumors has never been demonstrated by vaccination alone. A major reason could be that T cells are not sufficiently stimulated, and this is supported by the observation that general activation of antigen presenting cells enhance tumor protection. 4-1BB Ligand (4-1BBL) is an inducible co-stimulatory molecule necessary for proper T-cell expansion and treatment with an agonistic anti-4-1BB antibody has been found to eradicate established subcutaneous tumors in mice.

Materials and methods: Here, we investigated the effects of 4-1BBL expression into replication deficient adenovirus-based IiGP vaccine vector on the T cell immune responses in mice with and without tumor as well as in tumor protection.

Results: We show that this vaccine was as efficient as Ad5IiGP in stimulating naïve TCR transgenic CD8+ T cells, and there were no significant differences in the immune response induced by these two vaccines, in mice. In contrast, in tumor bearing mice, Ad5-IiGP-4-1BBL was found to decrease the CD8+ T cell expansion as compare to non-tumor bearing mice. This difference did not alter the therapeutic efficacy, however when 4-1BBL was added to a less immunogenic vaccination vector, Ad5-GP, the therapeutic anti-cancer activity was completely lost.

Conclusions: This new strategy does not improve vaccine efficacy. The reason for the apparent reduction in efficacy warrants further studies.

P1766

Treatment of experimental tumors with polymer-bound cytotoxic drugs revealing anti-tumor and immunomodulatory activity

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Purpose/Objective: Cancer treatment with established low-molecular-weight drugs can induce severe side effects, including impairment of anti-cancer immune responses. It was already demonstrated that these drugs, when bound to a polymer carrier display anti-cancer activity devoid of systemic toxicity. We previously documented that *N*-(2-hydroxypropyl) methacrylamide (HPMA)-based prodrugs bearing doxorubicin or taxanes exhibit direct anti-cancer effect as well as immunomodulatory activity, leading to development of tumor-specific resistance.

Materials and methods: High-molecular-weight (Mw $\sim$ 250 kDa) polymer carriers were designed in order to achieve water-soluble prodrugs with tunable drug content and high accumulation in the tumor. Conjugates binding either doxorubicin (Dox) or docetaxel (Dtx), or combination of both drugs were prepared. The drugs were bound to the carrier via hydrazone linkage, allowing for liberation of active drug in a pH-dependent manner and resulting in killing of the target tumor cells. The anti-cancer activity was studied in experimental models (EL4 T cell lymphoma in C57BL/6 mice, or 4T1 mammary carcinoma in BALB/c mice). The tumor resistance was proved in animals with completed tumor regression by a second challenge without any further treatment.

Results: Treatment of the murine tumors with the conjugates containing Dox or Dtx led to complete regression of EL4 and 4T1 tumors and development of treatment-dependent tumor resistance. The effective doses of the conjugates did not induce any apparent side toxicities. The Dox-containing conjugates induced immunogenic cancer cell death, already described for the free Dox, such as translocation of calreticulin to the cellular membrane and HMGB1 release. During the therapy, downregulation of MDSC was detected both in the tumor tissue and in the periphery. Interestingly, the conjugate containing both drugs showed higher anti-cancer activity when comparing with a mixture of two conjugates containing either Dox or Dtx, suggesting synergy between the drugs.

Conclusions: HPMA-based conjugates designed for increased tumor accumulation, bearing Dox and Dtx, are potent inducers of solid tumor regression and immunomodulatory activity, resulting in development of treatment-dependent immune-mediated tumor-specific resistance.

P1767

Two step selection protocol based on the expression of T cell activation marker CD137 increases efficacy in generating high-affinity allo-HLA restricted T cells

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Purpose/Objective: The broad application of adoptive immunotherapy targeting tumor-associated self-antigens is often hampered by the lack of an effective immune response targeting these antigens. As a consequence of self-tolerance high-affinity T-cells targeting self-antigens are often lacking. An attractive strategy is to exploit the immunogenicity of foreign leukocyte antigen molecules to generate an effective immune response against these antigens. Here we aimed to develop a protocol to efficiently isolate high-affinity peptide-specific allo-HLA restricted T-cells targeted to the B-cell compartment.
Materials and methods: A set of 18 novel HLA-A*0201 and B*0701 epitopes, encoded by B-cell specific genes, was selected from a large scale peptide-elution library. MHC-tetramers were generated for all peptides and used to isolate antigen-specific T-cells from HLA-mismatched PBMC donors. Expanding cultures were analyzed by multi-color combinatorial-coding MHC-tetramer screen and hundreds of clones were generated by FACS sort. Subsequently these clones were screened for exclusive B-cell recognition using a broad panel of various hematopoiesis restricted target cells. In a second isolation step MHC-tetramer positive T-cell lines were co-cultured with a CD40L-activated HLA-A*0201/B*0702 positive B-cell line. Following isolation of reactive T-cells based on CD137 expression, activated T-cells were clonally expanded and tested again for their recognition pattern.
Results: The implementation of the CD137 based selection step led to an increased isolation of high-affinity peptide-specific allo-HLA restricted T cells unparalleled by the isolation protocol lacking the selection procedure. In addition, the CD137 based selection step led to the isolation of novel high-affinity peptide-specific allo-HLA restricted T-cells targeting antigens against which no reactivity could be elicited previously.
Conclusions: Applying the methods of this study we were able to generate large numbers of high-affinity B-cell specific allo-HLA restricted T-cells including some CD79B reactive candidates. However, no T-cells suitable for adoptive T-cell therapy could be isolated. We speculate that implementing an additional selection step separating T-cells exerting unwanted reactivity from T cell with a desired recogniton pattern will further increase the selection efficiency and lead to the isolation of therapeutically relevant T-cells.

P1768

Virus-like particle mediated delivery of a-galactosylceramide improves the generation of anti-tumour responses

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Purpose/Objective: Virus-like particles (VLP) derived from the Rabbit Hemorrhagic Disease Virus (RHDV) are highly efficient vehicles for delivery of antigen to antigen-presenting cells (APC), however VLP do not provide the stimulatory signals required to generate optimal cytotoxic T lymphocytes (CTL). The glycolipid α-galactosylceramide (α-GalCer) is a potent stimulator of a subset of CD1d-restricted T cells, natural killer T (NKT) cells. Recognition of α-GalCer by NKT cells promotes production of interferon-γ and CD40/CD40L interactions that can provide an adjuvanting boost, assisting in APC priming of CTL. This research developed a composite particle of VLP with α-GalCer (VLP/α-GalCer).
Materials and methods: C57BL/6 mice ($n = 10$) were vaccinated intra-venously (i.v.) with 100ug of VLP/α-GalCer, α-GalCer or VLP alone. Splenocytes were isolated 24 h later and cell populations were analysed by flow cytometry for their activation status. In further studies mice were vaccinated (i.v.) with α-GalCer, VLP/α-GalCer or VLP alone and the ability to resist B16 tumour challenge was determined by inhibition of growth of tumours and by induction of cytotoxic T cells. Finally we assessed whether the production of IgG to the parent VLP could enhance the uptake of VLP *in vivo* and increase the overall ability of mice to resist tumour challenge.
Results: VLP/α-GalCer significantly increased the level of dendritic cell activation, inflammatory cytokine production, promoted antibody class switching and protected against growth of subcutaneous tumour. HPLC analysis of VLP/α-GalCer estimated that a 100 μg of VLP/α-GalCer was associated with ~20 ng of α-GalCer. Interestingly, VLP/α-GalCer was able to stimulate CTL responses equivalent to a 100 μg of VLP + 200 ng of unassociated α-GalCer.
Conclusions: This data demonstrates a 10-fold benefit to the adjuvanting activity of α-GalCer when it is associated with the VLP. Co-delivery of antigenic peptides and adjuvants on the scaffold of a VLP provides promise as a novel immunotherapeutic for the treatment of cancer.

Poster Session: Natural Products and Plant Medications

P1769

Effects of heat-killed Lactobacillus on allergic immune responses in a murine model of asthma

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Purpose/Objective: *Lactobacillus* NTU101 (NTU101) is a Taiwan-native strain of lactic acid bacteria isolated from human infant feces. It resists gastric acid and bile salts and has immunomodulatory capacity. In this study, we investigated the effects of heat-killed NTU101 with inulin (NTU101 sample) on allergic and gut immune responds.
Materials and methods: BALB/c mice sensitized intraperitoneally and challenged with ovalbumin (OVA) were oral administrated with NTU101 sample for 22 weeks. The effects of NTU101 on airway hyperresponsiveness (AHR), airway inflammation, serum antibody levels, natural killer cell activity and cytokine secretions from splenocytes, mesenteric lymph node and Peyer's patch were evaluated.
Results: NTU101 supplementation reduced the levels of inflammatory mediators, such as interleukin IL-4, IL-6, and TNF-a in bronchoalveolar lavage fluid (BALF) when compared with those of the control group. Feeding NTU101 sample can increase the levels of IL-2 secretion from ConA-stimulated splenocytes, mesenteric lymph node, and Peyer's patch. It also significantly increased IFN-g secretions from ConA-stimulated splenocytes, and Peyer's patch. Further, oral administration of NTU101 sample can reduce IL-5 and IL-10 secretions from ConA-stimulated splenocytes, and increase IL-10 secretion from ConA-stimulated mesenteric lymph node.
Conclusions: Although NTU101 can't improve AHR, it can alleviate bronchoalveolar inflammation, and has the immune tendency toward Th1 response.

P1771

Immunomodulating effects of exopolysaccharides produced by a cyanobacterium from the Blue Lagoon in Iceland

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Purpose/Objective: The Blue Lagoon is located on the geothermally active Reykjanes peninsula in Iceland. Beneficial effects of regular bathing on psoriasis, a T cell mediated disease, were discovered shortly after its formation in 1976. The Blue Lagoon coccoid cyanobacterium *Cyanobacterium aponinum* is a dominating member of the microbial ecosystem of the Blue Lagoon geothermal seawater, where it releases polysaccharides into its surroundings (exopolysaccharides, EPS). The aim of the study was to investigate whether EPS isolated from cultured *C. aponinum* affected maturation of human dendritic cells (DCs) and their ability to activate allogeneic $CD4^+$ T cells *in vitro*.
Materials and methods: Human monocyte-derived DCs were matured in the absence or presence of EPS (0.1−100 μg/ml) and the effect determined by measuring cytokine secretion by ELISA and expression of surface molecules by flow cytometry. Furthermore, DCs matured in the presence of EPS at 100 μg/ml were co-cultured with allogeneic $CD4^+$ T cells and the effect determined by measuring cytokine prodcution by ELISA and flow cytometry and expression of surface markers and transcription factors by flow cytometry.
Results: Human monocyte-derived DCs matured in the presence of EPS at 100 μg/ml secreted increased levels of IL-10 compared with DCs matured without EPS, whereas no changes were observed in IL-6 and IL-12p40 secretion. Allogeneic $CD4^+$ T cells co-cultured with DCs that had been matured in the presence of EPS secreted increased IL-10 levels compared with allogeneic $CD4^+$ T cells co-cultured with DCs matured without EPS, without any difference in secretion of IFN-γ, IL-17 and IL-22. There was a tendency towards increased frequency of $Foxp3^+$ and $IL\text{-}10^+$ $CD4^+$ T cells and decreased frequency of $PD1^+$ $CD4^+$ T cells when they were co-cultured with DCs matured in the presence of EPS.
Conclusions: EPS isolated from *C. aponinum* stimulate DCs to secrete increased levels of IL-10 without affecting secretion of IL-6 or IL-12p40. In co-culture, these DCs induced allogeneic $CD4^+$ T cells to secrete IL-10, and to upregulate Foxp3 and reduce PD1 expression, a phenotype resembling Tregs. This raises the question whether treatment in the Blue Lagoon induces upregulation of Tregs, which in turn could suppress the overly active T cells in inflammatory skin diseases like psoriasis.

P1773

Pharmacological characteristics of *Polygonum viscosum* Buch.-Ham. ex D. Don and its application in immunological disorders

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Purpose/Objective: Scientists are searching for new leads from the natural sources to combat different disorders. Until recently an insignificant part of the plants has been scientifically evaluated for their medicinal values. The studies were undertaken to discover new drugs from the natural sources. *Polygonum viscosum* Buch.-Ham. ex D. Don belongs to the plant family Polygonaceae Juss. and is widely distributed throughout Bangladesh. It is locally known as Bish-katali. *Polygonum viscosum* Buch.-Ham. ex D. Don is used for the remedy of killer diseases as well as debilitating diseases. The studies were leads to the development of a range of secondary metabolites of diversified structural types such as flavonoids, terpenoids, and cinnamic acid derivatives.
Materials and methods: The structures of the isolated compounds were elucidated mainly by high field Nuclear magnetic resonance (NMR) and other spectroscopic techniques.
Results: Quercetin 3-O-(6'-feruloyl)-β-D-galactopyranoside was found to exert a mild anticholinergic activity. A total of fifteen compounds were isolated, of which six compounds viz. viscozulenic acid, viscosumic acid, viscoazucine, viscoazulone, viscoazusone, and quercetin 3-O-(6'-feruloyl)-β-D-galactopyranoside appeared to be novel natural products. From the *in vivo* cytotoxicity assay of the novel compounds against two cancer cell lines (LOX and OVCAR-3), only quercetin 3-O-(6'-feruloyl)-β-D-galactopyranoside was found to be active against ovarian cancer cell line (OVCAR-3) with an IC_{50} value of 13.26 μg/ml. In the *in vitro* anti-HIV-1 assay, viscoazulone and quercetin 3-O-(6'-feruloyl)-β-D-galactopyranoside exhibited anti-HIV-1 activity at IC_{50} value of 33.13 μg/ml and 25.61 μg/ml, respectively, while no such activity was observed with other compounds.
Conclusions: The studies will increase the potentially of *Polygonum viscosum* Buch.-Ham. ex D. Don for the production of various pharmaceutical raw materials and new drugs of worldwide to a large extent.

P1774

Phytochemical isolated from *Ramalina farinaceae* showed anti-RSV activity and immune modulatory effects

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Purpose/Objective: Respiratory syncytial virus (RSV) is a member of the Paramyxoviridae family. Disease arising from RSV infection in humans is driven by a combination of host immune alteration and extensive viral replication. Phytochemical extracts of *Ramalina farinaceae* (*Rf*) was analyzed on the inhibitory effect on RSV infectivity/replication, and extractive-induced immune modulation.
Materials and methods: *Ramalina farinaceae* was screened for anti-RSV and immune modulatory activities using a combination of standard viral plaque reduction systems, and FACS-based cell proliferation assays.
Results: *Ramalina farinaceae* inhibited RSV in a dose-dependent manner with IC_{50} 22.45 μg/ml. Screening for immune modulation was analyzed using naïve splenocytes from BALB/c mice. *Rf* extracts showed to have no effect on CD4+ or CD8+ T-lymphocytes nor dendritic cell activation analyzed by a FACS based cell proliferation assay. However a stimulation and expansion of the B-lymphocytes population could be observed. Further screening of Sekikaic acid, a pure chemical compound isolated from *Rf* showed a fourfold enhancement of anti-RSV activity (IC_{50} 5.69 μg/ml). Time-of- addition studies showed that *Rf* and Sekikaic acid inhibited RSV at a post-entry stage of the virus replication cycle.
Conclusions: A compound Sekikaic acid isolated from *Rf* together showed high anti-RSV activity, and promising immune modulatory effect. This remains to be proven in *in vivo* studies.

P1775

Screening chemical libraries for immunosuppressive compounds

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Purpose/Objective: The purpose of this study was to identify novel compounds with immunosuppressive activity by *in vitro* screening of chemical libraries using method called Fluorescent Cell Chip (FCC).
Materials and methods: Previously described EL-4 murine T cell derived reporter cell lines stably expressing transgenes with promoter regions from mouse β-actin, IL-2, IL-4, IFN-γ and IL-10 and ORF for EGFP were employed in the study. In the first round of screening 1120 compounds from chemical library of marketed drugs and 500 compounds from library of flavonoids were incubated respectively at 0.4 and 50 μg/ml for 20 h with reporter cell line for IL-2 either activated with PMA/ionomycin or not. Following incubation cell viability and EGFP-mediated fluorescence was assessed by flow cytometry. Compounds that caused the most significant decrease in fluorescence while not significantly affecting viability were further analyzed. In the second round of screening dose response effect of selected substances on viability and EGFP-mediated fluorescence of all five reporter cell lines was assessed. The tested concentration range was 0.05−3.2 μg/ml for marketed drugs and 1.56−50 μg/ml for flavonoids.
Results: Screening of the library of marketed drugs allowed for identification of 11 drugs that could possibly exert immunosuppressive action. Among drugs that inhibited EGFP-mediated fluorescence of PMA/Ionomycin activated EL-4-derived cell lines in FCC test there were compounds already used as immunosuppressants (e.g. cyclosporine) as well as several drugs used in treatment of diseases not related to immune system, such as anthelmintic agent niclosamide or respiratory inhibitor and antifungal and antibacterial agent antimycin A. Screening of flavonoid library by means of FCC test identified 50 compounds that inhibited EGFP-mediated fluorescence of several EL-4-derived cell lines. Structure-dependence was observed.
Conclusions: FCC has been shown to be a good tool for the screening for immunosuppressive compounds as in a screening of chemical library of marketed drugs it was able to 'rediscover' immunosuppressive drugs, such as cyclosporine. In this study we have identified several naturally occurring flavonoids and their derivatives as compounds that potentially could exert immunosuppressive action on T cells.

P1776

The anti-inflammatory effects of Bitter Gourd between adipocytes and macrophages

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Purpose/Objective: Obesity is associated with a state of low-grade chronic inflammation. Many studies have demonstrated that increased infiltration of macrophages is characterized in the obese adipose tissue, suggesting that a paracrine loop between adipocytes and macrophages causes an inflammatory vicious cycle. Bitter gourd (*Momordica Charantia*) is a tropical vegetable that has also been used in a traditional medicine for treating diabetes. The ethyl acetate extracts of Bitter gourd powder (EA-BGP) has been shown to have anti-inflammatory effects, but the influence on macrophages and adipocytes is unclear.
Materials and methods: We investigated the EA-BGP on inflammatory changes in differentiated 3T3-L1 adipocytes and Raw 264.7 macrophages. The pro-inflammatory cytokine secretions were examined after 24 h treatment with EA-BGP. To elucidate the interaction between adipocytes and macrophages, Raw 264.7 cells were pretreated with different concentrations of EA-BGP and co-cultured with 3T3-L1 cells.
Results: We showed that EA-BGP decreased the production of MCP-1 and IL-6 in 3T3-L1 adipocytes. TNF-α and IL-6 secretions in LPS-stimulated Raw264.7 was also inhibited. Coculture of 3T3-L1 adipocytes and RAW 264.7 macrophages markedly enhanced the production of MCP-1 and IL-6 compared with control cultures. However, the production of these cytokines was decreased when Raw264.7 were pretreated with EA-BGP.
Conclusions: This study provides evidence for the anti-inflammatory effect of EA-BGP on macrophages and adipocytes. The inflammatory changes induced by the interaction between adipocytes and macrophages were also improved by the pretreatment of Raw264.7 with EA-BGP. The ethyl acetate extracts of bitter gourd may have potential to ameliorate the inflammation in obese adipose tissue.

Poster session: Neonates – Knowledge and Interventions

P1777

Acetaminophen application during murine pregnancy triggers metabolic and immunological changes in dams and impairs fetal development

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Purpose/Objective: Recently, human studies revealed a significant correlation between acetaminophen (APAP; e.g. paracetamol, tylenol) use during pregnancy and an increased risk of asthma development in the children later in life. Insights on how APAP interferes with maternal adaptation to pregnancy and fetal development are still elusive. Therefore, we studied the effects of prenatally APAP-induced liver injury on immunological parameters in non-pregnant and pregnant females as well as fetal development.
Materials and methods: Therapeutic (0–150 mg/kg) or toxic doses (250–450 mg/kg) of APAP were injected i.p. into non-pregnant and pregnant mice on gestation day (gd) 12.5. Liver injury was quantified by measurement of plasma transaminase activity (ALT) 24 h (gd 13.5) or 96 h (gd 16.5) after APAP challenge. The maternal immune cell profile was analysed by flow cytometry. Embryo implantation and abortion rate, fetal body weight and total cell numbers of fetal liver and thymus as well as hematopoetic stem cell (HSC) counts were calculated.
Results: Pregnant mice were more susceptible to APAP-induced liver injury compared to non-pregnant mice indicated by significantly elevated transaminase activities and displayed increased frequencies of hepatic NKT and activated T cells. APAP challenge had no effect on implantation and abortion rate on gd 13.5 or gd 16.5. However, fetal development was delayed in prenatally APAP-challenged mice mirrored by reduced fetal body weight. The number of total cells in liver and thymus as well as percentage and absolute number of hematopoietic stem cells within the liver was significantly reduced in fetuses from APAP-treated mice compared to non-treated ones.
Conclusions: Pregnant mice are more susceptible toward APAP-induced liver damage. Prenatally induced liver injury severely impairs fetal development along with damage of the liver progenitor cell reservoir and primary lymphoid organs. Interestingly, the differences between pregnant and non-pregnant mice were particularly profound at the limit from therapeutic to toxic APAP doses. We now intend to identify how maternal APAP-medication causes liver damage and deregulation of maternal and fetal liver progenitor cell reservoir, as this may be an early indicator of APAP-induced increase of children's risk for allergic or autoimmune diseases.

P1778

Adapting vaccine formulation to the neonatal context is required to obtain safe protection against the respiratory syncytial virus

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Purpose/Objective: The human respiratory syncytial virus (hRSV) is the leading cause of severe bronchiolitis in infants worldwide. The most severe RSV diseases occur between 2 and 6 months-of-age, so pediatric vaccination will have to be started within the first weeks after birth, when the immune system is prone to Th2 responses that may turn deleterious upon exposure to the virus. So far, the high risk to prime for immunopathological responses in infants has hampered the development of vaccine.
Materials and methods: In the present study we investigated the safety and efficacy of ring-nanostructures formed by the recombinant nucleoprotein N of hRSV (N^{SRS}) as a mucosal vaccine candidate against RSV in BALB/c neonates, which are highly sensitive to immunopathological Th2 imprinting.
Results: A single intranasal administration of N^{SRS} with detoxified *E. coli* enterotoxin LT(R192G) to 5–7 days old neonates provided a significant reduction of the viral load after an RSV challenge at 5 weeks of age. However, neonatal vaccination also generated an enhanced lung infiltration by neutrophils and eosinophils following the RSV challenge. Analysis of antibody subclasses and cytokines produced after an RSV challenge or a boost administration of the vaccine suggested that neonatal vaccination induced a Th2 biased local immune memory. This Th2 bias and the eosinophilic reaction could be prevented by adding CpG to the vaccine formulation, which, however did not prevent pulmonary inflammation and neutrophil infiltration upon viral challenge.
Conclusions: In conclusion, protective vaccination against RSV can be achieved in neonates but requires an appropriate combination of adjuvants to prevent harmfull Th2 imprinting.

P1780

Deficient IFN-gamma response to *Mycobacterium tuberculosis* antigens in infants improved since 1 year of age

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Purpose/Objective: How interferon (IFN)-γ responses mature in early age is poorly known. Here we analysed IFNγ response to *Mycobacterium tuberculosis* antigens across different ages and different clinical conditions.
Materials and methods: Two hundred and twenty-seven immunocompetent children (<15 years old) were included. 30 were not infected despite TB contact. Fifty-one presented TB disease, 40 latent TB and 106 had clinical signs mimicking TB disease but alternative diagnosis. 23 HIV-infected children were also analysed. The Tuberculin-Skin Test (TST) consisted in intradermal injection of 5 IU PPD (Tubertest Aventis Pasteur MSD, Lyon, France). The IFNγ release assay QuantiFERON-TB gold In-Tube assay (QF-TB-IT, Cellestis, Ltd, Australia) was used. QF-TB-IT specificity was defined as the proportion of negative results identified within uninfected cases. QF-TB-IT sensitivity was defined as the proportion of positive results within TB disease cases.
Results: In immunocompetent children: (1) QF-TB-IT specificity was 100%; (2) low sensitivity of QF-TB-IT in infants (40%) increased with aging (77% and 85% in $1 \leq 5$ years and in ≥ 5 years subgroups respectively); (3) in Latent TB, agreement between TST and QF-TB-IT was 0% in infants but 40% in $1 \leq 5$ years and 65% in ≥ 5 years groups; (4) combining QF-TB-IT and TST improved diagnosis of both TB disease and Latent TB; (5) finally, indeterminate results were <5% in children exposed to TB (TB-disease, Latent TB and uninfected-contact) but 24% in young children with TB excluded (bacterial pneumonitis in most instances).

In HIV-infected children, indeterminate results ($n = 5/23$) were mostly observed at diagnosis of symptomatic HIV infection (4/5). 6/23 children were co-infected with *M. tuberculosis*. Immunological results

were $TST^-/QF\text{-}TB\text{-}IT^+$ in 2/6, $TST^+/QF\text{-}TB\text{-}IT^-$ in 1/6, $TST^+/QF\text{-}TB\text{-}IT^+$ in 1/6 and $TST^-/QF\text{-}TB\text{-}IT^-$ in 2/6.

Conclusions: Impaired IFNγ secretion limits the use of IFNγ release assays in <1 year old children and likely leads to high risk of progression *in vivo*. Combining TST and QF-TB-IT improved diagnosis in ≥1 year old children especially in HIV infected children. Indeterminate results were mostly observed in bacterial pneumonitis unrelated to TB. Studies are in progress to address the mechanisms of IFNγ inhibition by ongoing bacterial infections.

P1781

Dose sparing effect of the adjuvant covaccine HT on neonatal immune responses to a whole H5N1 influensa vaccine from cell culture

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Purpose/Objective: Influenza pandemics are associated with high mortality and morbidity rates. The objective of this study was to evaluate the immunogenicity of a whole H5N1 virus influensa vaccine (WIV) produced in cell culture, in a neonatal murine model. Furthermore, to access whether the adjuvant CoVaccine HT could enhance neonatal immune responses to WIV.

Materials and methods: NRMI mice (1 week) were vaccinated in the scapular girdle with increasing doses of H5N1 WIV (HA 0.1; 0.25; 0.5, 1 μg) w/wo 0.2 mg (1st imm) and 0.5 mg (2nd imm) of Covaccine HT and boosted 16 days later. Blood was obtained from the tail vein before the 2nd immunization and weekly thereafter. WIV specific IgG antibodies were measured by ELISA. A Hemagglutination inhibition assay using horse RBC was used to measure the HAI titers.

Results: The H5N1 WIV was immunogenic in neonatal mice, all WIV-vaccinated groups had significantly higher IgG anti-WIV than unvaccinated controls. Co-administration of CoVaccine HT with WIV significantly enhanced IgG anti-WIV response and 0.5 μg HA along with 0.2/0.5 mg CoVaccine HT elicited significantly higher IgG levels than the optimal HA dose of 5 μg HA alone ($P = 0.0148$) after one vaccination. WIV elicited dose dependantHAI titers. Thus, mice that recived 0.5 μg HA + 0.2/0.5 mg CoVaccine HT had significantly higher HAI titers than mice that received 0.1 μg HA ($P = 0.0024$) or 0.25 μg HA ($P = 0.0369$) with the same CoVaccine HT schedule. A low WIV dose of0.5 μg HA given with 0.2/0.5 mg CoVaccine HT elicited significantly higher HAI titers than 1 μg ($P = 0.0335$) and 5 μg ($P = 0.003$) HA alone.

Conclusions: The results demonstrate that the WIV H5N1 vaccine is immunogenic in neonatal mice. WIV-specific Abs and HAI titers were significantly enhanced by CoVaccine HT, and a 10-fold dose reduction could be achieved compared to non adjuvanted WIV. The results encourage us to further study CoVaccine HT«s mechanisms of action and its dose sparing effects on neonatal immune responses to WIV.

P1782

Phagocytosis of *Escherichia coli* and *Staphylococcus aureus* by monocytes and neutrophils from septic and healthy preterm and term newborns

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Purpose/Objective: Because of the immature immune function, the newborn is susceptible to intestinal and systemic infections. *Escherichia coli* and *Staphylococcus aureus* are prevalent infectious agents in neonatal intensive care units, responsible for outbreaks of sepsis. The aim is to investigate whether cord blood monocytes and neutrophils have an impairment of development in its ability to phagocytize Gram-positive and negative rods, compared to septic neonates and adults.

Materials and methods: Monocytes and neutrophils from umbilical cord blood of healthy preterm <34 weeks of gestation (Group 1), ≥34 and <37 weeks of gestation (Group 2) and term (Group 3) newborns, and from peripheral blood of term newborns diagnosed with late-onset sepsis and healthy adults, used as controls, were exposed to propidium iodide-labeled *E. coli* and *S. aureus* to analyze their phagocytic capacity using flow cytometric method.

Results: Compared with adults, monocytes and neutrophils from extremely preterm neonates revealed decreased phagocytic capacity, with the exception of *S. aureus* by neutrophils. Monocytes and neutrophils from late preterm neonates showed decreased phagocytic ability only for *E. coli*. Term newborns demonstrated similar phagocytic ability compared with adults. Regarding neonates with sepsis, neutrophils exhibited a marked decrease in phagocytic capacity for both *E. coli* and *S. aureus*, but monocytes showed equivalent results, when compared to adults.

Conclusions: Until now, these data demonstrate that the observed impairment in the intake of *E. coli* and *S. aureus* by phagocytes is correlated with gestational age at birth. In addition, the diminished phagocytic ability of neutrophils from septic neonates could explain the inability of these newborns to control infections.

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P1783

Phenotypic differences in leukocyte populations among septic and healthy preterm and full-term newborns

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Purpose/Objective: Evaluate the absolute numbers of dendritic cells (DCs), monocytes, T and B lymphocytes and the frequencies of myeloid (mDCs) and plasmacytoid (pDCs) DC subtypes and T and B lymphocyte subsets in cord blood of healthy preterm and term newborns and in peripheral blood of septic newborns.

Materials and methods: The absolute numbers and frequencies of DCs, monocytes, T and B lymphocytes and their subsets were determined in umbilical cord blood of healthy preterm <34 weeks of gestation (Group 1, $n = 5$), ≥34 and <37 weeks of gestation (Group 2, $n = 8$) and term (Group 3, $n = 16$) newborns, and in peripheral blood of term newborns diagnosed with late-onset sepsis ($n = 23$), all of them analyzed by flow cytometry. In septic neonates, leukocyte populations were determined at time of diagnosis. Sepsis was diagnosed by a positive blood culture and/or accompanied by

compatible signs and symptoms of clinical sepsis at a mean age of 25.3 days.

Results: The results showed that the absolute numbers of circulating DCs ($Lin^-HLA\text{-}DR^+$) were similar between all groups, as well as the frequency of DC subtypes, mDCs ($CD11c^+/CD123^-$) and pDCs ($CD11c^-/CD123^+$) and immature mDCs and pDCs ($CD11c^+CD1a^+$ and $CD123^+CD1a^+$, respectively). Monocyte ($CD14^+HLA\text{-}DR^+$) numbers were equivalent among the groups and further analyzed regarding the expression of activation markers CD80 and CD86. The frequencies of monocytes $CD80^+$ were equivalent among the groups and, regarding monocytes $CD86^+$, the frequency in septic neonates was lower compared with groups 2 and 3. T $CD3^+CD4^+$ lymphocyte numbers did not differ among the groups, as well as the frequencies of naïve, central memory, effector memory and effector T $CD4^+$ subsets. The $CD19^+$ B lymphocytes were present in equivalent numbers among the groups. The frequency of naïve B cells ($CD19^+CD27^-$) was significantly higher in group 3 and, inversely, the frequency of memory B cells ($CD19^+CD27^+$) was significantly lower in group 3 compared with all the other groups.

Conclusions: In general, the frequencies of DC subtypes, as well as, monocytes, T $CD4^+$ and B subsets were not affected by both the clinical condition of sepsis and the chronological age of the newborns.

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P1784

Polysaccharide-induced hyporesponsiveness in neonatal mice depends on the polysaccharide type

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Purpose/Objective: We have shown that neonatal vaccination with a serotype 1 pneumococcal polysaccharide (PPS) induces hyporesponsiveness to serotype 1 pneumococcal conjugate vaccine (PCV) and meningococcal C PS induces hyporesponsiveness by depleting MenC-PS specific B-cells. 23valent PPS (PPS23) induces hyporesponsiveness to 4 of 6 serotypes of a 7-valent PCV (Prevnar), while for 2 serotypes PCV7 could overcome the hyporesponsiveness.

The aim of the study was to assess if neonatal PPS23-vaccination induces hyporesponsiveness to PCV10 (Synflorix), that has protein carriers that differ between serotypes and from PCV7.

Materials and methods: Neonatal NMRI mice (1 week old) were primed with 1/5 dose of PPS23 or saline and boosted 16 days later with 1/4 human dose of PCV10 (serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F) or saline. Blood samples were taken weekly from 2-weeks and at 12 weeks. IgG antibodies (Ab) to 7 PCV10 serotypes were measured by ELISA. The number of Ab secreting cells (AbSC) specific for serotypes 4, 9V and 18C was enumerated in spleen of mice primed with PPS-23 or saline and boosted with PCV10.

Results: Mice primed with PPS-23 as neonates and boosted with PCV10 as infants mounted significantly lower IgG response to 3 of 7 PCV10 serotypes measured (serotypes 1 $P < 0.001$, 4 $P = 0.001$, 6B $P = 0.021$, ns for 9V, 14.18°C, 19F) compared to mice primed with saline. Neonatal PPS23-priming also significantly reduced the number of PCV10-induced AbSC specific for the two of three serotypes tested (serotypes 4 $P = 0.01$, 9V $P = 0.3$, 18°C $P = 0.01$) compared to saline priming, suggesting that PPS23 has depleted B-cells specific for these serotypes.

Conclusions: Neonatal priming with PPS23 induces hyporesponsivenss to some of the PCV10 serotypes, which can be overcome by PCV10 for serotypes 9V and 14, as previously shown for PCV7 and for 18C as well. The results show that PPS-induces hyporesponsiveness in neonatal mice varies depending on the type of polysaccharide rather than the conjugate.

P1786

TLR-2 and TLR-4 mediated responses in monocytes from preterm and term newborns are distinct from those of adults

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Purpose/Objective: The neonatal immune system, particularly in very-preterm newborns, has been considered functionally immature and recent studies suggest that this susceptibility to infections may be due to functional alterations in antigen-presenting cells that can lead to secondary deficiencies in adaptive responses. The aim is to evaluate the activation and response of monocytes derived from umbilical cord blood of healthy adequate-for-gestational-age term and preterm newborns, after *ex-vivo* Toll-like receptor 2 and 4 (TLR-2 and TLR-4) stimulation.

Materials and methods: Cord blood mononuclear cells (CBMC) from healthy preterm <34 weeks of gestation (Group 1), ≥34 and <37 weeks of gestation (Group 2) and term (Group 3) newborns, and peripheral blood mononuclear cells (PBMC) from healthy adults were isolated by density gradient centrifugation. Adherent cells (monocytes) were stimulated with TLR-2 and TLR-4 agonists, Pam3CSK4 (5 μg/ml) and LPS (10 ng/ml), respectively, for 24 h at 37°C. The functional outcome of the stimulation was determined by pro- and anti-inflammatory cytokine secretion in the supernatants (measured by cytometric bead array) and the expression profile of activation markers on monocytes exhibiting normal forward- and side-scatter properties by flow cytometry and staining normally for CD14 and HLA-DR.

Results: After LPS or Pam3 stimulation, interleukin (IL)-8, IL-6, TNF-a and IL-1β production by monocytes was substantially higher in term and both preterm groups when compared with adults, but inversely, IL-10 production was lower in the three groups of newborns compared to adults. Regarding the expression profile of activation markers on monocytes after stimulation with Pam3 or LPS, very-preterm newborns from Group 1 showed reduced expression of HLA-DR, CD80 and CD86 when compared with the other groups.

Conclusions: So far, our data suggest that neonates present greater capacity of developing an inflammatory response, which gets even more accentuated by the apparent lower production of IL-10. These results indicate an imbalance in cytokine production in the neonatal period which can increase the susceptibility to develop sepsis.

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Poster Session: Nutrition and Vitamins – Basic Immunology as a Foundation for Intervention

P1788

Consumption of *Bifidobacterium animalis* subsp. lactis Bi-07 in a clinical trial enhances ex vivo phagocytic activity in healthy elderly adults

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Purpose/Objective: Immunosenescence and ageing have been associated with changes in gut microbiota composition, susceptibility to infections, and metabolic disorders. Potential functional foods to counteract immunosenescence are probiotic bacteria and prebiotic complex oligosaccharides that have been shown to beneficially modulate gut microbiota and to improve immune function.
Materials and methods: A randomized double-blinded placebo-controlled cross-over human clinical trial was designed (ClinicalTrials.gov Identifier: NCT01586247) where 37 healthy elderly volunteers consumed probiotic *Bifidobacterium animalis* subsp. *lactis* Bi-07 (Bi-07, 10^9 cfu/day), prebiotic galacto-oligosaccharides (GOS, 8 g/day), their synbiotic combination (Bi-07 + GOS), or placebo (maltodextrin, 8 g/day). Cytokine production of LPS stimulated whole blood cultures were quantificated by multiplex ELISA. Cellular innate immune function was determined *ex vivo* by quantification of phagocytosis and oxidative burst activity in mono- and granulocytes. Gut microbiota composition and metabolic activity was determined using 16S rRNA sequencing and short-chain fatty acid quantification from fecal samples.
Results: Consumption of Bi-07 improved phagocytic activity of granulocytes *ex vivo* ($P < 0.001$), while decreasing the percentage of granulocytes showing oxidative burst activity ($P = 0.06$). LPS induced cytokine production in whole blood showed reduction of IL-1beta concentration in probiotic group ($P = 0.03$). Analyses of fecal samples showed that consumption Bi-07 significantly increased the total number of bacteria ($P = 0.046$) and number of *Bifidobacterium animalis* subsp. *lactis* ($P = 0.03$).
Conclusions: Consumption of probiotic Bi-07 may be beneficial for improving gut and immune system function in healthy elderly adults and could thus counteract the detrimental effects of immunosenescence. Probiotics may provide a cost-effective solution to improve quality of life in the elderly population.

P1789

Folate deficiency elevates inflammatory responses but lowers Th1 and Treg responses *in vivo*

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Purpose/Objective: The aim of the study is to investigate the effects of folate deficiency on T-cell and inflammatory responses.
Materials and methods: Two groups of 7-weeks-old Foxp3-eGFP reporter mice were fed an AIN-76 diet containing either 0 (F0) or 2 (F1) mg folic acid per kg diet for 13 weeks.
Results: After 3 weeks of the folate deficient diet feeding, the serum folate concentrations of F0 mice decreased significantly. Total $Foxp3^+$ Treg % and $CD25^+$ $Foxp3^+$ Treg % of $CD4^+$ T cell in spleen, and total $Foxp3^+$ Treg % in Peyer's patch (PP) decreased significantly in the F0 group. Moreover, Th1 cytokine IFNγ, Treg-related cytokine IL-10 and Th2 cytokine IL-4 secretions from splenocytes of the F0 group after ConA stimulation decreased significantly, but there were no significant differences in other Th2 cytokines such as IL-5 and IL-13. However, TNFα produced by LPS-stimulated peritoneal excudate cells (PEC) was significantly increased in the F0group.
Conclusions: Folate deficiency may lower Th1 and Treg responses but enhance inflammatory responses *in vivo*.

P1790

IL-1, IL-6 concentration and apoptosis induction in BALB/c 3T3 cells after simultaneous incubation with iron and chromium

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Purpose/Objective: Chromium and iron are essential microelements playing a vital role in many cellular processes. The aim of this study was to examine the effect of simultaneous treating with chromium and iron on IL-1α, IL-6 concentration and the effect of induction of apoptosis on BALB/c 3T3 cells.
Materials and methods: Cells were cultured as adherent monolayers in plastic tissue-culture dishes in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum and antibiotics. The cells were cultured on 96-well dishes (2×10^5 cells/ml) in medium. After 24 h of incubation, the medium was exchanged for fresh DMEM (control), DMEM supplemented with 50, 100, 300, 500 and 700 μM $CrCl_3$, 50, 100, 300, 500 and 700 μM $FeCl_3$, one supplemented with 50 μM $CrCl_3$ and 500 μM $FeCl_3$ or 50 μM $FeCl_3$ and 500 μM $CrCl_3$. After 24 h of incubation cytokines concentration and apoptosis were measured according to the original manufacture's instruction.
Results: Iron and chromium used separately increase statistically significant IL-1α concentration after incubation with iron chloride or chromium chloride, whereas they decrease statistically significant IL-6 concentration when compared with control cells. Moreover, chromium decreases statistically significant number of apoptotic cells, while iron increases apoptotic cells. Simultaneously, incubation with 50 μM $CrCl_3$ and 500 μM $FeCl_3$ decreases statistically IL-1α concentration and the number of apoptotic cells when compared with cells incubated with iron chloride at the concentration of 500 μM $FeCl_3$. Moreover, a statistically significant decrease was observed in IL-6 concentration when compared with cells incubated with control cells and fibroblasts incubated with 50 μM $CrCl_3$ or 500 μM $FeCl_3$. Simultaneously, the incubation with 50 μM $FeCl_3$ and 500 μM $CrCl_3$ increases statistically significant IL-1α concentration and the number of apoptotic cells when compared with control cells and cells incubated with iron chloride at the concentration of 50 μM $FeCl_3$, whereas it decreases IL-6 concentration when compared with control cells.
Conclusions: The increase of IL-6 concentration after the simultaneous treatment with chromium and iron suggests the synergistis interaction between these elemnts.

P1791

Impact of coffee galactomannans on Pneumococcal vaccine response in a preclinical model

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Purpose/Objective: Antibody (Ab) responses to vaccination are a useful measurement in the assessment of immune status, especially in suspected immune deficiency. Although Pneumovax (pneumococcal polysaccharide (PPS) vaccines) are safe and effective in reducing the

incidence of pneumococcal invasive disease in healthy adults, they are weakly immunogenic in children <2 years old and in the elderly, the two groups at higher risk. The new generation vaccine Prevenar® 13 has coupled the PPS to a protein carrier, converting the PPS to T cell-dependent type of antigens (Ag), characterized by affinity maturation and extensive Ab subclass switching. Previous studies have shown that nutritional intervention is able to increase the specific Ab response to Pneumovax vaccine. Coffee infusion polysaccharides are composed mainly by galactomannans, on average they account for 68%. Acetylated mannans from coffee have shown immunostimulatory activities in an experimental model, illustrated by the activation of B-and T-lymphocytes. The aim of this study was to evaluate whether coffee galactomannans modulate Pneumococcal vaccine response.

Materials and methods: Balb/c female mice (6 weeks old) were fed *ad libitum* 1% galactomanans from coffee or control diet through pelleted chow during 3 weeks before immunization with Prevenar® 13 (200 μl subcutaneously) and maintained in the same diet for four additional weeks. In order to evaluate the specific antibody response to the vaccine, blood samples were collected just before immunization and 28 days after. Levels of specific IgG Ab anti-PPS in sera were assessed by ELISA.

Results: Among the 13 PPS present in the vaccine, animals responded to 10 PPS at different percentages: PPS4, PPS5, PPS19A, PPS18C, PPS3, PPS6B, PPS19F, PPS9V, PPS1 and PPS7F, showing PPS4 and PPS5 the highest percentage of responders. In fact, significant increases in the IgG anti-PPS4 and PPS5 titers were observed at day 28 post-immunization. A slight increase was observed for PPS19A and PPS18C, whereas no effect was shown for the other PPS.

Conclusions: The present results show for the first time that coffee galactomannans are able to increase the systemic Ab responses to a conjugated Pneumococcal vaccine, encouraging the possibility to use them as bioactive polysaccharides.

P1792

Sulforaphane induces neutrophil death and alters the mechanism of death in a dose dependent manner with implications for resolution of inflammation

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Purpose/Objective: Neutrophil (PMN) apoptosis is an essential component of inflammatory resolution. Sulforaphane (SFN) in cruciferous vegetables has been demonstrated to have therapeutic potential for tumours as an inducer of apoptosis at high doses in neoplastic cells. Here we test the hypothesis that SFN induces PMN death. The objective is to investigate the impact of dietary SFN doses on PMN viability, and determine whether the mode of death is apoptotic.

Materials and methods: PMN from healthy volunteers were cultured for 20 h with SFN (5–40 μM) and inhibitors of different modes of PMN death including wortmanin (100 nM), Z-VAD FMK (20 μM) and necrostatin (25 μM). Apoptosis was measured by flow cytometry using annexin V PI and JC-1 staining. Morphological markers of cell death were observed with Diff-Quik and MitoTracker staining.

Post-hoc pairwise comparisons were conducted using one-tailed Dunnett's test. Significance was taken as $P < 0.05$.

Results: SFN 5 μM increased PMN apoptosis, mean annexin V PI determined viability (APV) of cells reduced from 23.1% to 14.7% ($n = 28$, $P = 0.044$). Apoptosis as the primary form of death is supported by morphological markers and successful inhibition of SFN 5 μM's action by general caspase inhibitor Z-VAD FMK. Mean APV was not reduced by the addition of SFN 5 μM to Z-VAD FMK 20 μM treated cells.

Paradoxically, higher doses of SFN (40 μM) increased APV to 39.0% ($P < 0.001$). However JC-1 staining showed an altered profile of reduced yellow and green fluorescence, mitotracker showed oncotic mitochondria (fig.1) and Diff-Quik staining showed condensed nuclei in oncotic cells. Consequently we believe that the absence of positive annexin fluorescence is due to alternative modes of death involving cell swelling and failure to express phosphatidylserine. SFN 40 μM's effects were undeterred by Z-VAD FMK 20 μM, necrostatin 25 μM and wortmanin 100 nM suggesting that the mechanism of death is not caspase dependent, necroptotic or autophagic.

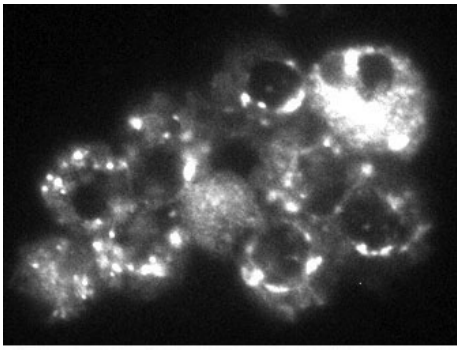

Fig 1. MitoTracker labelled 20h PMN treated with SFN 40uM

Conclusions: SFN increases PMN apoptosis at low dietary doses, therefore as apoptosis is anti-inflammatory low dose SFN may have a therapeutic role. However at higher doses an alternative mode of death is induced that may not be anti-inflammatory, for instance necrosis can perpetuate inflammation. Further research is required to elicit the mechanism occurring and its inflammatory significance.

P1793

The immuno-regulatory effects of olive oil in murine model

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Purpose/Objective: The quantity of dietary lipid ingestion has increased during the past decade, which inducing the development of many disease. It has been know that the quality and quantity of dietary oil could regulate immune responses. Furthermore, in recent years the olive oil was the first choice for cooking oil in Taiwan. In general, the immunomodulatory effects of dietary lipid were investigated the diet rich in *n*-3 or *n*-6 polyunsaturated fatty acids, but less attention has been paid to the effects of monounsaturated fatty acid (MUFA). In order to study the regulatory effects of MUFA-rich diet on the immune responses, we like to use a murine model.

Materials and methods: The BALB/c mice were fed with AIN-76 diet that contain 5% or 20% olive oil for 8 weeks.

Results: The results showed that body weight, feed efficiency, serum IgM, IgE and total antibody levels were higher than control group, especially in 20% MUFA group. Otherwise, mice fed with 5% olive oil significantly reduce the amount of PGE2 production from peritoneal cells, IL-2 and IFNγ secretion form splenocytes. In addition, olive oil increased TGF-β1 production, which was negatively correlated with IL-2 secretion.

Conclusions: These results indicated that MUFA-rich could regulate T cell cytokines secretion. And the inhibition of lymphocyte activity might correlate with regulatory T cells activation.

Poster Session: Targeting Bacterial, Fungal and Parasitic Infections

P1794

Differential mechanisms of resistance to pulmonary *Aspergillus fumigatus* infection in immunocompetent rats

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Purpose/Objective: Immunity to pulmonary infection by *Aspergillus fumigatus* is investigated mainly in immunosuppressed mice in settings of lethal outcome, but there are few data concerning nonlethal infection as well as antifungal responses in other animal species. The aim of this study was to examine immune mechanisms of resistance of immunocompetent rats to nonlethal *A fumigatus* infection by analyzing response in two rat strains (Dark Agouti/DA/and Albino Oxford/AO/) known to differ in the type of reactivity to variety of stimuli.

Materials and methods: Antifungal pulmonary response (1×10^7 conidia, intratracheally) was investigated by: (1) fungal elimination, (2) the type of cytokine response (IFN-γ, IL-17, IL-4) measured in lung homogenates (overall response) and in cells from different compartments (draining lymph nodes/DLN/, recovered from lungs by enzymatic digestion/lung cells/ and bronchoalveolar lavage/BAL/), and (3) fungicidal capacity (lung cells).

Results: Progressive decrease in fungal burden was observed in both rat strains, but more efficient in DA rats (fungal elimination at day 15 versus day (d) 30 in AO rats). Conidia removal took place in proinflammatory *milieu*, as increased IFN-γ and IL-17 content in lung tissue (significantly higher in DA rats) and unchanged IL-4 (both strains) was observed. Increased DLN activity (cellularity, proliferation) was observed in both strains, but differences between these strains were noted in dynamic/intensity of IFN-γ (two phases of increase, day 3 and day 15, in DA*vs* continuous, day 3 to day 15, in AO) and magnitude of IL-17 production (significantly higher in DA rats). Later during infection (d 15) higher production of proinflammatory cytokines by BAL cells was observed in AO rats. Similar IFN-γ production was noted by lung cells in both strains at all time points, with higher levels of IL-17 in AO rats from day 7 to day 15. However, more pronounced fagocytic and fungicidal activity was observed in DA rats.

Conclusions: Differences in induction and expression of cytokine response and effector activities may contribute to differences in the efficacy of *A. fumigatus* conidia elimination in these strains.

P1795

Expression of virulence-related genes of Brucella melitensis and *B. abortus* in murine macrophages

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Purpose/Objective: Brucellosis is a worldwide-distributed zoonosis. *Brucella* evades the immune response and survives inside the macrophages by using different mechanisms. Finally, the bacteria are located into membrane vesicles in between the endoplasmic reticulum. A better understanding of the molecular mechanisms that lead the intracellular trafficking may help to explain one of the main *Brucella* attributes of virulence. The aim of the present study was to follow the expression of virulence-related genes of *B. melitensis* and *B. abortus* strains along their intracellular trafficking in murine macrophage, and correlate it with the virulence of the strain.

Materials and methods: Complementary probes for the mRNA of the *sodC*, *omp25* and *bvrR/bvrS* genes and for the 16S rRNA as positive control were synthesized coupled to different fluorochromes at 5«-OH. *Brucella*-infected J774A.1 macrophages were hybridized with the fluorescent probes at different time points post-infection and observed under the confocal microscopy. The macrophages were also stained with specific antibodies to markers of the membrane vesicle-containing *Brucella* to trace the bacteria traffic.

Results: The *sodC* and *omp25* genes were expressed into early endosomes by both, virulent and no virulent strains. However, the expression of the *bvrR/bvrs* genes was diminished in the early endosomes, and thereafter peaked along the intracellular trafficking in vesicles pertaining to the endocytic pathway, in a way dependent of the virulence of the strain.

Conclusions: The products of the genes expressed differentially appear to be related with the pathway and fate of *Brucella* within the host cells.

P1796

Oxidative modification of lipoprotein by periodontopathic bacteria

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Purpose/Objective: Periodontal disease is a highly prevalent disorder affecting up to 90% of the global population. Several studies support an association between periodontal disease and atherosclerosis with a crucial role for the major periodontal pathogens. We previously reported that *Porphyromonas gingivalis* (*P. g.*) or *Aggregatibacter actinomycetemcomitans* (*A. a.*) accelerated atherosclerotic plaque formation in hyperlipidemic apoE−/− mice by initiating inflammation. Because oxidative modification of lipoproteins plays a major role in atherosclerosis, the present study was designed to test the oxidative activity of *P. g.* or *A. a.* on low-density lipoprotein (LDL).

Materials and methods: Atherosclerotic plaque formation in the aortic sinuses of Apoe^{shl} mice i.v. challenged with *P. g.* 381 or *A. a.* HK1651 was assessed by oil red-O staining. Anti-mouse Hocl-oxidized LDL and 4HNE antibodies were used for immunohistochemistry. Detection of intracellular ROS generation was performed using H2DCF-DA. Quantitative RT-PCR was performed using primers specific for LOX-1, NOX-1, NOX-2, NOX-4, p22phox, p47phox and β-actin. Oxidation of LDL by monocytes exposed to bacteria was assayed by ox-LDL ELISA kit.

Results: *P. g.*- or *A. a.*-challenge markedly induced ox-LDL and 4HNE positive areas in proximal aortic lesions. TLR-2, LOX-1 and NADPH oxidase subunit-specific mRNA levels were significantly increased. Furthermore, *P. g.* exposure caused monocytes to oxidize LDL in a dose dependent manner. Inhibition of gingipain with KYT-1 (Arg-gingipain inhibitor) or KYT-36 (Lys-gingipain inhibitor) significantly suppressed the modification of LDL by *P. g.*

Conclusions: These results suggest that *P. g.* or *A. a.* promotes the oxidation of LDL and contributes to atheroma development. This study was supported by a grant (no.22390398) from the Ministry of Education, Science, Sports and Culture, in Japan.

P1798

Preliminary evaluation of suppressive activity of rHsp60_Pb18 in the immune response in experimental paracoccidioidomycosis

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Purpose/Objective: Paracoccidioidomycosis (PCM), caused by the dimorphic fungus *Paracoccidioides brasiliensis*, is a chronic, granulomatous and progressive disease. PCM has been associated with various degrees of suppressed cell-mediated immunity. Previous data from our group has shown that complete Freund's adjuvant (CFA) induced a protective effect in mice previously infected with *P. brasiliensis*. However, the beneficial effect was utterly reversed when CFA was emulsified with a fraction containing fetuin-binding proteins (FBP) from *P. brasiliensis*.

Materials and methods: The identification of FBP was done by in-gel digestion with trypsin and nLC-MS-MS peptide analysis. The heterologous protein expression was obtained in *Escherichia coli* transformed with pET28a subcloned with the *HSP60* gene of *P. brasiliensis* strain 18. To evaluate the possible role of Hsp60 in suppressing immune response, recombinant protein (rHsp60_Pb18) wascultured with spleen cells under polyclonal stimulation of T (ConA) or B (LPS) cells or popliteal lymph nodes cells from ovalbumin (OVA)-immunized mice stimulated with OVA.

Results: We identified the major protein 60-kDa and two minor 58 and 50-kDa proteins that comprise the FBP fraction as heat shock protein 60 kDa from strain 18 of *P. brasiliensis* (Hsp60_Pb18). When spleen cells of male BALB/c mice were incubated with spleen cells stimulated with mitogens (ConA or LPS) in the presence of different rHsp60_Pb18 concentrations, we observed that 50 μg of rHsp60_Pb18 induced a remarkable decrease in the number of lymphocytes when compared to control cells. Significant decrease of lymphocyte number was also observed with mitogen-stimulated culture treated with 10 μg of Hsp60_Pb18. Similar results were obtained when we carried out an assay of antigen-specific proliferation by using popliteal lymph node cells.

Conclusions: These results suggested that Hsp60_Pb18 can induce cell death when lymphocytes are activated. This actvity of Hsp60_Pb18 might be important in *P. brasiliensis* virulence, based on which we suggest that the death of lymphocytes can be involved in immune suppression seen in PCM. Other assays *in vitro* and *in vivo* are being conducted to determine the suppressive activity of rHsp60_Pb18 in the immune response.

P1799

Surface layer proteins as important virulence factors in *Clostridium difficile* infection

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Purpose/Objective: *Clostridium difficile* (*C. difficile*) is a nosocomial pathogen and the cause of *Clostridium difficile* infection (CDI) in hospitals worldwide. In recent years, hypervirulent strains have emerged, resulting in increased mortality rates amongst those suffering from CDI. The bacteria possess a surface layer composed of two distinct Surface Layer Proteins (SLPs), High Molecular Weight (HMW) and Low Molecular Weight (LMW) proteins. These SLPs are important virulence factors that play a role in adherence and evasion of the immune system. We have previously shown that ribotype 001 evokes an immune response by activating both innate and adaptive immunity. The purpose of this study was to determine if SLPs from different PCR ribotypes induced different immune responses.

Materials and methods: In this study we have used molecular evolutionary tools to analyse HMW and LMW proteins of the SLP from a diverse range of *C. difficile* ribotypes. We have prepared a multiple sequence alignment and phylogenetic tree, and examined the selective pressure variation across ribotypes for both LMW and HMW proteins. We examined ribotypes 001, 002, 005, and 017 for the effect of SLPs on the activation of bone marrow derived dendritic cells (BMDCs) and macrophages by Enzyme-linked immunosorbent assay (ELISA).

Results: Positive selective pressure is synonymous with protein functional shift and we have found evidence of positive selection in both the HMW and LMW proteins in different ribotypes. Mapping the signatures of positive selection onto of the phylogeny of the ribotypes revealed a single branch separating those ribotypes with positive selection in the HMW protein from those with positive selection in the LMW protein. While all ribotypes induced cytokine production in immune cells, with some upregulation of IL-1β, IL-6, IL-10, IL-12p40, IL-23, TNF-alpha and IL-12p70, the strength of the response differed with some only weakly inducing these cytokines. Indeed, the SLPs least related to ribotype 001 showed the strongest activation.

Conclusions: The results of the bioinformatics study were indicative of a potential correlation between positive selection and the ability of the ribotype to elicit an immune response. This is seen in the diference in response strength of immune cells to SLPs from different ribotypes. By analysing the ribotypes that show the greatest and weakest response in immune cells, and comparing those results with our evolutionary analysis, we can now determine functionally relevant residues for the induction of an immune response.

P1800

T cells specific for the *Aspergillus* proteins Crf1 and catalase1 develop in patients recovering from invasive aspergillosis

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Purpose/Objective: Impaired T cell mediated immunity may play a role in the increased risk of aspergillosis, a severe complication of allogeneic stem cell transplantation (alloSCT). To allow development of new therapeutic strategies including adoptive transfer of antigen specific T cells, we investigated the role of *Aspergillus*-specific T cells in the clearance of aspergillus infection.

Materials and methods: We analyzed the T cell response against *A. fumigatus* in seven patients who had received an alloSCT because of a hematological malignancy and were diagnosed with invasive aspergillosis 1−10 months after SCT. PBMC were stimulated with the overlapping peptides of Crf1 and Catalase1 at several time points before and after the diagnosis of aspergillosis and analyzed by flowcytometry using intracellular staining for IFNy and CD154.

Results: When PBMC were stimulated with Crf1 and Catalase1 and restimulated with Crf1 and Catalase1 after 7 days, we detected clear populations of *Aspergillus*-specific T cells in six out of six patients with regression of aspergillus lesions or stable disease. In these six patients a peak of Crf1- and Catalase1-specific CD154+ CD4+ T cells was present around the time of regression of aspergillus lesions. The peak frequency of Crf1- and Catalase1-specific CD154+ T cells varied between 0.5% and 3% of CD4+ T-cells. In most patients both Crf1- and Catalase1-specific T cells were present. After clearance of the infection the frequency of *Aspergillus*-specific CD4+ T cells decreased to low or undetectable levels. In one patient no Crf1- or Catalase1-specific T cells could be identified. This patient had progressive aspergillus infection while suffering from severe GvHD of the skin, liver and colon and died shortly after the diagnosis of invasive aspergillosis.

Conclusions: Using only 2 *A. fumigatus* proteins as target antigens, we demonstrated the induction of *Aspergillus*-specific T cells in six patients, coinciding with the decline of aspergillus lesions. These data indicate that an immune response directed against *A. fumigatus* proteins helps to clear an aspergillus infection. Adoptive T cell therapy with *Aspergillus*-specific T cells generated *in vitro* by stimulating with *A. fumigatus* proteins like Crf1 and Catalase1, may therefore be an attractive strategy to treat patients with invasive aspergillosis.

Poster Session: Targeting Virus-Induced Diseases

P1802

Novel frequently recognized T-cell epitopes from adenoviral species C restricted to HLA-B*44

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Purpose/Objective: Adenoviral infections are common and widespread in a civilian population. Besides the mild and self-limiting infections, adenovirus is described in immune compromised individuals to lead to severe pathogenesis with a mortality rate of up to 76%. The most prevalent species are HAdV 2 and HAdV 5 of Mastadenovirus serotype C.

Adenovirus can establish a persistent presence in its host for a long time after clinical symptoms have subsided which could be an explanation for endogenous virus infection in patients with impaired immune system. Besides, low T cell counts have been shown to correlate with severity of infection and mortality.

Thus, immunotherapeutic strategies using adoptively transferred AdV-specific T cells appear promising.

Materials and methods: The prevalence of adults in the general population that have endured adenoviral infection in their life and therefore possess memory CTL varies between 65% and 100%. Accordingly a sufficient amount of PBMCs of healthy donors exhibit significant proliferative responses to HAdV antigens. In this study we follow the strategy of reverse immunology to identify CTL epitopes of several adenoviral proteins restricted by HLA-B*44. Epitopes predicted by SYFPEITHI were screened for recognition by PBMCs from HLA-matched healthy donors using IFN-y-ELISPOT after 12 days of pre-stimulation. Specificity and HLA restriction were confirmed by tetramer staining. Candidate epitopes were tested on at least fifteen donors to characterize their dominance and frequency of recognition and further examined for multifunctional immune response via intracellular cytokine staining and flow cytometry. Furthermore, cytotoxicity assays were performed and promiscuity of the T cell epitopes was determined by testing non-HLA-B*44 and HLA-B*44 supertype donors for reactivity.

Results: Fifteen peptides from HAdV antigens were positive by IFN-y-ELISPOT, two of them with high frequency of recognition (>60% of tested PBMCs) and multifunctional T-cell response. In addition, several less frequently recognized peptides were identified which induced promiscuous responses within the HLA-B*44 supertype.

Conclusions: The novel identified frequently recognized epitopes (FREPs) will be relevant for immunotherapy using autologous adenovirus-specific memory T cells or for vaccination strategies in order to cope with fatal HAdV infections.

Poster Session: Transplantation

P1803

A role of anti MICA antibodies in humoral rejection mechanisms after kidney transplantations

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Purpose/Objective: Allogeneic transplantations are connected with a risk of a rejection of the transplanted organ. Rejections can be mediated by cellular or humoral mechanisms or by both of them. It is well established that humoral mechanisms are based on the induction of antibodies in a recipient that recognise miss-matched HLA-antigens of a donor. However, there are some reports that anti-MICA antibodies take part in the rejection mechanisms too. The aim of our work was to investigate if anti class I and class II HLA and anti MICA antibodies, respectively, are present in our group patients who underwent the kidney transplantation.

Materials and methods: Forty-nine kidney transplanted patients who developed antibody dependent rejection were investigated. The rejection was verified by biopsy * by detection of C4d fragments of the complement system in histological samples. Antibodies against MICA antigens were analysed by multiplex analysis (LUMINEX) using diagnostic kits 'LABScreen Mixed Class I and II' and 'LABScreen MICA Single Antigen' from the OneLambda Company. We established also specificities of the MICA antibodies, i.e. which MICA antigens they recognise.

Results: Anti MICA antibodies were detected in 13 patients (26.5%). However, 10 out of them (77%) possessed also anti HLA antibodies (both, anti class I as well as class II). We suppose that in the patients left, i.e. in three (23%) who were anti MICA positive, however anti HLA negative, the anti MICA antibodies were responsible for the antibody dependent rejection mechanisms. By investigating specificities of the detected anti MICA antibodies, we found that they recognised mostly MICA18 and MICA01 antigens. Sera of patients with MICA antibodies were usually polyspecific, only 30% of them were directed against one or two MICA antigens, all others reacted with three or more. We didn't find a correlation between the specificity of MICA antibodies and MICA molecules of the donor.

Conclusions: MICA antibodies are induced after allogeneic kidney transplantations either together with anti HLA antibodies or also independently from them. As such patients underwent the humoral type of rejection, it can be supposed that anti MICA antibodies are responsible for.

P1804

Analysis of polyclonal antithymocyte globulin preparations

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Purpose/Objective: Antithymocyte globulins (ATGs) are purified IgG fractions derived from rabbits immunized with the Jurkat T cell line (ATG-Fresenius) or human thymocytes (Thymoglobulin). They are widely used as strong immunosuppressive agents for prevention and treatment of allograft rejection episodes and graft versus host disease. The clinical efficacy of different batches of ATGs could be potentially influenced by differences in the targeted antigens or in the amount of leukocyte reactive antibodies.

Materials and methods: Four batches of ATG-Fresenius and Thymoglobulin were compared regarding their capacity to interact with human leukocytes from transplant recipients and healthy donors. The reactivity of ATG preparations with Jurkat cells and with human leukocytes was analysed by flow cytometry. Additionally, ATGs derived from different batches were probed with a panel of cell lines expressing high levels of ATG antigens. Furthermore, the ability of ATGs to mediate complement-mediated lysis of human monocytes and lymphocytes was also compared.

Results: The high conformity in ATG preparations of both manufacturers found in our study makes variations of different batches of ATGs in respect of their clinical efficacy unlikely.

Conclusions: The methods described in this study allow for a comprehensive evaluation of ATG preparations in clinical use regarding their antibody composition and their ability to mediate lysis of human leukocytes.

P1805

Anti-HLA antibodies monitoring in kidney transplant recipients: high prevalence of donor specific anti-HLA class II antibodies

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Purpose/Objective: Anti-HLA antibodies post transplant follow up has been suggested by several studies as a useful tool to identify patients with acute or chronic rejection risk, and therefore useful to implement the necessary therapeutic measures that would help us minimize its clinical impact.

Materials and methods: We had carried out a transversal and prospective study of 304 kidney recipients. Anti-HLA antibodies were tested using Luminex technology, screening and single antigen (Genprobe). The median number of tests performed per patient was 3.3. Single antigen with MFI > 1500 and specificity against any HLA donor antigen, determined by HLA-A/B/DR typing or linkage disequilibrium when HLA-DQB1* was not available, was assigned as donor specific antibody (DSA). De novo anti-HLA II DSA were tested for complement-fixing HLA antibodies using a C1q single antigen bead assay (One Lambda).

Results: Post transplant anti-HLA antibodies were detected in 109 patients (38.8%): 29% (28/109) were antibodies anti-HLA class I and II, 9.2% (10/109) were anti-HLA class I and 65.1% (71/109) were anti-HLA class II.

Within the 109 positive patients, 35 (32%; 35/109) were de novo and most of them anti-HLA class II (34/35; 97.1%). De novo anti-HLA class II antibodies were DSA in 27 patients (77%; 27/35), anti-DQB1* was the most frequent specificity (81%; 22/27), anti-DR was found in five patients (18.5%; 5/27) most of them anti-DRB4* (3/5). DSA anti-DR and anti-DQB1* was only present in one patient. Anti-DP antibodies were not considered as we lacked typing data. Within the 22 anti-DQB1* patients, 16 (73%; 16/22) were de novo complement-fixing DSA as detected by the C1q assay.

Fifteen patients from the group with de novo anti-DQB1* DSA (55.5%; 15/27) suffered a rejection episode, causing graft failure in four recipients who failed to respond to therapy. The low number of cases does not allow us to determine whether complement-fixing DSA might influence evolution or therapy response.

Conclusions: Anti HLA antibodies monitoring allowed us to discover a high frequency of de novo anti-HLA class II DSA in kidney recipients, most of them against HLA-DQB1* specifities. These patients were found to have a higher rejection incidence. HLA-DQB1* typing should be recommended in donors and patients in order to take its compatibility into account.

P1806

Antibodies against intracellular antigens develop as a result of tissue damage induced by donor lymphocyte infusion after allogeneic stem cell transplantation

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Purpose/Objective: Allogeneic hematopoietic stem cell transplantation (alloSCT) is an effective treatment for hematological malignancies. Graft-versus-host disease (GvHD), however, often is a life-threatening complication. To reduce GvHD incidence and severity, T-cells can be depleted from the stem cell graft and re-administered later as donor lymphocyte infusions (DLI) to preserve graft-versus-tumor (GvT) effect.

Whereas T-cells are key mediators of clinical responses (GvHD and GvT) after alloSCT, the role of antibody responses is still largely unknown. Antigens encoded by the Y-chromosome (histocompatibility-Y or H-Y antigens) have been described as targets for T-cells and antibodies in female-to-male transplantation. In this study, we investigated the dynamics and specificity of antibody responses against H-Y antigens and their X-encoded homologs during treatment with T-cell depleted alloSCT and DLI.

Materials and methods: Two broadly expressed intracellular H-Y antigens, ZFY and DBY, and their X-variants were produced in two overlapping protein fragments in *E. coli* and coupled to fluorescent beads. In a high-throughput Luminex bead assay, 1300 serum/plasma samples collected from 38 female and 54 male patients with acute myeloid leukemia or myelodysplastic syndrome during treatment with alloSCT and DLI were screened for antibody binding to antigen-coupled beads. Mean fluorescence signals were normalized for unspecific binding as defined by signal on a bead coated with an irrelevant viral protein.DLI.

Results: In 5/17 (29%) female-to-male transplantations, high titer antibodies reactive against DBY ($n = 2$) or ZFY ($n = 3$) developed shortly after DLI and were accompanied by antibodies binding to the respective X-variant. Three of these patients experienced extensive GvHD after DLI, leading to major tissue damage in the affected organs. Signal on the X-antigen could be fully blocked using the homologous Y-antigen as cold target, whereas *vice versa* signal on the Y-antigen could not always be inhibited by the X-antigen, indicating antibody binding to polymorphic (Y-specific) as well as monomorphic (X/Y-shared) epitopes.

Conclusions: Our data suggest that antibodies develop against intracellular H-Y antigens and their X-homologs when they are released in the extracellular milieu after tissue damage induced by T-cell mediated GvL or GvHD responses after DLI.

P1807

Blocking dendritic cell maturation with Mitomycin-C yields tolerogenic cells: studies of human cells *in vitro* and of rat cells in a heart allograft model

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Purpose/Objective: We aim to generate suppressive cells for tolerance induction in clinical organ transplantation. As immature dendritic cells (DC) have immune inhibitory functions we tried to block the maturation of human monocytes to DC with Mitomycin C (MMC), a chemotherapeutic drug. The resulting cells were analyzed *in vitro*. The action of corresponding rat cells was studied in a heart allotransplant model.

Materials and methods: Human monocytes were incubated with MMC, matured to DC, analyzed by qPCR for immunosuppressive molecules and tested in T-cell proliferation assays. Replacing monocytes with peripheral blood mononuclear cells (PBMC) would be more practical for clinical use. The tolerogenic action of PBMC was tested, in a heterotopic DA (donor) to PVG rat (recipient) heart transplant model. Recipients were injected with MMC-treated donor PBMC and transplanted with a heart from the same donor. Tolerance was defined as graft survival of >70 days.

Results: (1) MMC treatment inhibits the maturation of human monocytes to DC. (2) The cells express slightly increased levels of mRNA for arginase-1, iNOS, NOX2, TGF§, TNF-α and strongly increased levels for CEBP§, IL-10 and COX2 * all potential mediators of immunosuppression. (3) DC generated from MMC-treated monocytes suppress the primary T-cell response. Suppressed T-cells can not be restimulated. (4) Similar results are obtained with MMC-treated PBMC. (5) Like in humans, rat monocytes treated with MMC give rise to suppressive DC. (6) In a transplant model donor MMC-treated PBMC injected into recipients significantly prolong heart allograft survival (65 ± 17 versus 8.6 ± 0.3 days), 50% of animals becoming tolerant. The effect is abolished when monocytes are removed from PBMC. (7) Tolerant recipients reject third-party allografts indicating donor-specific suppression. (8) Tolerant rats display an increased number of CD4+CD25+ FoxP3+ cells in peripheral lymphoid organs. (9) Tolerance can be transferred with blood cells to naïve syngeneic animals.

Conclusions: MMC-treated monocytes or PBMC matured to DC suppress T-cell responses. The effect is due to arrest of DC maturation and probably mediated by suppressive molecules. MMC-treated donor PBMC injected into recipients induce Tregs and lead to long-lasting allograft survival. This model has clinical relevance.

P1808

Blood miR-142-5p expression in renal transplanted patients exhibiting chronic antibody mediated rejection and chronic allograft dysfunction

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Purpose/Objective: The unresponsiveness of patients undergoing chronic antibody mediated rejection (CAMR) to classical treatment

stress on the need for accurate biomarkers to improve its diagnosis. Because of their high stability, miRNA are pointed out as possible biomarkers.

Materials and methods: We performed expression profiling of miRNA in peripheral blood mononuclear cells from kidney transplanted recipients with CAMR, stable graft function, acute rejection episode, chronic graft dysfunction and renal failure. Our objectives were to study whether miRNA expression pattern may be associated to a diagnosis of CAMR and to determine if change in these miRNA may be a way to predict graft dysfunction.

Results: Among a total of 257 expressed miRNA and using non-parametric supervised analysis, we identified 15 miRNA differentially expressed in blood from patients with CAMR. Among them, we pointed on miR-142-5p, an hematopoietic specific cell lineage miRNA, significantly over-expressed in blood and biopsies from patients with CAMR. This miRNA was not increase in PBMC from patients with renal failure, suggesting that its over-expression is associated to immunological disorders in CAMR rather than to renal dysfunction. Furthermore, its expression is decreased in PHA-activated PBMC and not modulated in blood from patients with acute rejection, getting rid of an aspecific T cell activation effect in patients with CAMR. Finally, the absence of modulation of this miRNA in blood from patients under immunosuppression compared to healthy volunteers suggests that its expression is not influenced by the treatment. We then evaluated if its expression may be correlated with the risk of renal dysfunction in a cohort of 133 stable kidney transplant recipients among which 112 patients remained stable and 21 degrade their renal function in time. We identified two classes of patients according to the miR-142-5p expression, with a low (expression >0.73) and a high risk of degradation (expression<0.73). High risk patients had 2.2 times more risk of renal dysfunction (CI 95% = [0.85, 5.88], $P = 0.0991$). Finally, analysis of miR-142-5p predicted targets suggest that its over-expression in a situation of CAMR may be a way to control activation.

Conclusions: Our results suggest miR-142-5p could be used as a specific blood biomarker of CAMR and to predict chronic graft failure.

P1809

Circulating pro-inflammatory CD4+ CD28null T cells increase the risk for a cardiovascular event shortly after kidney transplantation

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Purpose/Objective: An unusual population of pro-inflammatory $CD4^+$ T cells that have lost the co-stimulatory molecule CD28 ($CD4^+CD28$null T cells) is expanded in patients with end-stage renal disease (ESRD). $CD4^+CD28$null T cells are associated with cardiovascular disease and may cause atherosclerotic plaque instability by their highly pro-inflammatory nature. In this study we tested the hypothesis that expansion of $CD4^+CD28$null T cells in ESRD poses a risk factor for an atherosclerotic vascular event (AVE) after kidney transplantation.

Materials and methods: In a prospective study the number of circulating $CD4^+CD28$null T cells was established in 295 ESRD patients, prior to receiving a kidney allograft. All patients were screened for a history of symptomatic atherosclerotic disease and routinely evaluated for cardiovascular disease (CVD) by a cardiologist before kidney transplantation. Besides age and gender, the traditional risk factors for atherosclerotic disease were recorded. During the first year after transplantation, patients were followed for the occurrence of an AVE.

Results: In 31% of all ESRD patients a medical history of CVD was present (CVDpos). The percentage (10.3% versus 5.6%, $P = 0.002$) and absolute number (38 versus 22×10^3/ml, $P = 0.01$) of $CD4^+CD28$null T cells were increased in CVDpos patients compared to patients without documented CVD (CVDneg). Within the first year after transplantation, an AVE occurred in 20 patients, five cases (2.3%) in the CVDneg group and 15 cases (18.3%) in the CVDpos group. Over 80% of all AVEs occurred within 3 months after transplantation with a median time from transplantation to event of 5 days. Univariate analysis showed that besides CVDpos (HR 8.1, $P < 0.001$), age (HR 1.04, $P = 0.02$), dyslipidaemia (HR 8.8, $P = 0.004$) and the % of $CD4^+CD28$null T cells (1.04 per % increase, $P = 0.01$) were significantly associated with the occurrence of a post-transplantation AVE. In a multivariate analysis, only CVDpos remained a significant risk factor with a significant and positive interaction between the terms CVDpos and the % of $CD4^+CD28$null T cells (HR 1.05, $P < 0.001$). Within the CVDpos group, the incidence of an AVE was 13% in the lowest tertile compared to 25% in the highest tertile of % of $CD4^+CD28$ null T cells.

Conclusions: The expansion of circulating $CD4^+CD28$null T cells is highly associated with the presence of CVD in ESRD patients and increases the risk for a cardiovascular event shortly after kidney transplantation.

P1810

CMV-specific CD8+ T-cell immunity is a co-factor for acute rejection in lung transplantation

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Purpose/Objective: Lung transplantation is the definitive treatment for terminal respiratory disease, but the associated mortality rate is high. Acute rejection of the transplanted lung is a key determinant of adverse prognosis. An epidemiological relationship has been established between acute lung rejection and CMV. However, the reasons for this association remain unclear.

Materials and methods: We performed a longitudinal study of CMV-specific T-cell responses and immune activation status, in PBMC and bronchoalveolar lavage fluid of lung transplant patients. Four groups were designated according to donor/recipient CMV serostatus: D^-/R^-, D^+/R^-, D^-/R^+ and D^+/R^+.

Results: Forty-four patients were included in this study. In D+ and D-/R- patients, in PBMC and in BAL, acute rejection was associated with higher levels of cellular activation (respectively $P = 0.0280$ and $P = 0.0143$). In D+/R- patients, CD38 expression on total memory $CD8^+$ T-cells was strongly correlated with those on CMV-specific $CD8^+$ T-cells ($r = 0.78$; $P < 0.0001$), and with the frequency of CMV tetramer$^+$ $CD8^+$ T-cells ($r = 0.63$; $P < 0.0001$).

CD38 levels on memory $CD8^+$ T-cells, frequency of CMV tetramer$^+$ were strongly correlated in blood and BAL from D^+/R^- patients ($r = 0.63$; $P = 0.012$ andr = 0.73; $P < 0.0001$), in contrast to D^-/R^- patients ($r = 0.08$; $P = 0.6$ and $r = 0.1$; $P = 0.7$).

We observed the same dominant clonotypes in CMV-specific $CD8^+$ T-cell populations from lung and blood, suggesting migration from periphery to CMV infected graft.

Conclusions: These findings provides further support that CMV-specific $CD8^+$ T-cell immunity links the observed relationship between CMV infection and the occurrence of acute lung rejection.

P1811

Comparative MHC region identity analysis between patients and HLA-identical unrelated donors in hematopoietic stem cell transplantation

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Purpose/Objective: In hematopoietic stem cell transplantation with HLA-identical unrelated donors, the risk of acute graft-versus-host disease is increased if there is incompatibility in the MHC haplotype as a whole, even in the presence of complete classical HLA loci identity. The aim of this study was to determine the degree of actual MHC haplotype identity between transplant patients and their HLA-identical unrelated donors.
Materials and methods: We studied 46 pairs of patient and HLA-identical unrelated donor from three hospitals (Vall d'Hebron, Germans Trias i Pujol, ICO-Duran i Reynals) in which a high-resolution study of 5 HLA loci (HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1) had been performed. Seven MHC region DNA microsatellites (STRs) were analyzed by multiplex PCR.
Results: The most conserved microsatellites were D6S273 and D6S265, both in the classical HLA gene region with 14 and 20 pairs fully matched and with only 0 and 1 couple mismatched, respectively. The least conserved microsatellites were located at the ends of the extended class I and II regions. The pair disparity rate varied between 5 out of 14 possible identities and 12 out of 14 possible identities (average, 7.8).
Conclusions: Globally, the MHC region identity rate between patients and their HLA-identical unrelated donors was 55%. Identity was particularly related to the classical HLA genes while disparity was higher at the extended regions.

P1813

Cyclosporine A impairs renal host defenses through NFATc1-dependent inhibition of Nod1-mediated bacterial phagocytosis

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Purpose/Objective: Urinary tract infections (UTI) and acute pyelonephritis (APN), mainly caused by uropathogenic *Escherichia coli* (UPEC), is the most frequent bacterial complication in renal transplant recipients receiving immunosuppressive treatment. However, it remains unclear how immunosuppressive drugs, such as Cyclosporine A (CsA), decrease renal resistance to UPEC.
Materials and methods: We use an experimental model of APN, administration of CsA and 11R-VIVIT, and SiRNA of *NFATc1*.
Results: Using an experimental model of APN, we show that mice treated with CsA exhibitgreater susceptibility to UPEC than vehicle-treated mice. The expression of the nucleotide-binding oligomerization domain 1 (Nod1), and neutrophil functions, including neutrophil migration capacity and phagocytic killing of *E. coli* were markedly reduced in CsA-treated mice infected by UPEC. *In vitro*, CsA markedly downregulated *Nod1* mRNA expression in neutrophils and macrophages. One of the main functions of calcineurin inhibitors is to impair the activation of the nuclear factor of activated T-cells (NFATs). Silencing the*NFATc1* mRNA isoform, similar to CsA, downregulated *Nod1* mRNA expression in macrophages, and administration of the 11R-VIVIT peptide inhibitor of NFATs to wild-type mice decreased their resistance to UPEC. Conversely, administration of synthetic Nod1 stimulating agonists, which partially restored *Nod1* mRNA expression without affecting NFATs inhibition caused by CsA, increased the resistance of CsA-treated mice to UPEC infection.
Conclusions: Collectively, these results indicate that CsA directly alters Nod1-mediated innate immune responses, and suggest that the prophylactic effects of Nod1 agonists might be helpful to reduce the incidence of bacterial infection of renal grafts.

P1814

Detection of rhesus cytomegalovirus as a viral reactivation marker in monkeys treated with immunosuppressants

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Purpose/Objective: Human cytomegalovirus (HCMV) is reactivated frequently in immunocompromised patients, such as transplant recipients. Transplant patients with CMV disease are likely to develop acute rejection after CMV infection or reactivation. The immunosuppressive regimens are usually used to overcome immunological barriers in transplantation, but they induce the reactivation of latent viruses. It is favorable to develop the immune control method for transplantation with minimal reactivation of latent infectious agents. Rhesus CMV (RhCMV) has a similar characteristics to HCMV and was adopted to study the virological and immunological responses in monkey with transplant.
Materials and methods: Total 8 Rhesus macaques (Macaca mulatta) were included in this experiment; five with low dose steroid treatment, and three with regimen accompanying depletion of immunocytes. All experiments were performed after receiving approval from the Institutional Review Board and the Institutional Animal Care and Use Committee and according to the National Institutes of Health guidelines. The quantification of RhCMV in blood was determined by real time polymerase chain reaction. Anti-RhCMV antibody titer was measured by indirect immunofluorescent staining using RhCMV 68-1-infected HEL299.
Results: All the monkey included in these experiments had IgG antibody to RhCMV with the titers from 1:80 to 1:1280. No or low level of RhCMV DNA was detected through 6 months in five monkeys, which were grouped into treatment with low dose of steroid before transplantation. Antibody titer to RhCMV in this group did not change at large. Other three monkeys were grouped with the aspect of applying immunosuppressant of immunocyte depletion. They were treated with the combination of the immunosuppressants, (Tacrolimus, Leflunomide, Mycophenolate mofetil (MMF) and Campath), (Tacrolimus, Leflunomide, MMF, Basiliximab and Rituximab), or (anti thymocyte globulin (ATG), anti-CD154, Leflunomide, FTY720 and Rapamycin). They all showed decreased lymphocyte counts and increased copy number of RhCMV, peaked $4600-7500/mm^3$ in blood, and minimal changes of anti-RhCMV antibody titers.
Conclusions: Strong immunosuppressants could induce the reactivation of latent virus and maybe do harmful effect on transplantaion. Therefore it is needed to adopt the mild immunosuppressant regimens or new methods to overcome the immunologic barrier and virologic reactivation.

P1815

Donor cell composition and reactivity can predict risk of acute graft-versus-host disease

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Purpose/Objective: Graft-versus-host disease (GVDH) is a potentially fatal complication after allogeneic hematopoietic stem cell transplantation (AHSCT). There are limited possibilities to predict the risk for this adverse reaction. The aim of this project was to design a functional strategy for assessing the probability of acute GVHD in each individual case.

Materials and methods: Blood samples were collected from patients and donors prior to AHSCT. Two groups of seven patients each were identified, one where all individuals developed moderate to severe acute GVHD and the other where none showed any clinical signs of GVHD. Peripheral blood mononuclear cells (PBMCs) isolated from each donor were cultivated in a mixed lymphocyte reaction (MLR) with cells from the corresponding patient. The cells were phenotypically characterized by flow cytometry before and after MLR. In addition, levels of 27 cytokines and granzyme-B in the surrounding medium were measured using Luminex multiplex assay and ELISA respectively.

Results: Donor cells prior to the MLR had significantly lower frequencies of $\gamma\delta$ T-cells and T-cells expressing the NK-cell markers CD56 and CD94 in the GVHD-group. Donor samples from this group also exhibited a lower proportion of T-cells that expressed CD95, compared to those from the control group.

Both groups had comparable frequencies of the major lymphocyte populations at the onset. However at day 6 of the MLR there was a significant difference in the distribution of CD4+ and CD8+ T-cells: the proportions in the GVHD groups were more or less unchanged (68% $CD4^+$/30% $CD8^+$) while the non-GVHD group showed a reversed ratio (26% $CD4^+$/43% $CD8^+$) ($P = 0.03$). The differences in frequencies remained significant for the naïve $CD4^+CCR7^+CD45RO^+$ T-cells ($P = 0.02$). Regarding levels of soluble factors in the MLR, we could only detect a trend towards lower concentrations of IFN-γ in the GVHD group ($P = 0.07$).

Conclusions: We conclude that differences in the composition of the allogeneic graft could be used to predict its allo-reactive potential. Donor derived $\gamma\delta$ T-cells and NKT cells could be beneficial for promoting peripheral tolerance and protecting against GVHD. It would also seem that expression of CD95 on donor T-cells might be coupled to lower allo-reactivity, possibly because these cells are more likely to be deactivated through apoptosis. Further, a relative increase in the naïve CD4+ T-cell population after allogeneic MLR might be predictive for acute GVHD *in vivo*.

P1816

During aGVHD low-dose IL-2 activates allogeneic T cells and fails to induce Treg-mediated tolerance

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Purpose/Objective: Acute graft-versus-host disease (aGVHD) is the main life threatening complication of allogeneic stem cell transplantation (allo-HCT). Recent clinical trials suggest that administration of regulatory T cells (Treg) can reduce GVHD but this approach needs optimization. An alternative is the use of interleukin-2 (IL-2) that can directly boost Treg *in vivo*. We have previously shown that a low-dose short course of IL-2 can cure type 1 diabetes (T1D) in mice by specifically activating Treg in the pancreas. Here, we tested this strategy in a mouse model of aGVHD.

Materials and methods: aGVHD was induced by grafting lethally irradiated [B6 × DBA/2] F1 mice with B6 bone marrow and B6 $CD3^+$ lymphocytes. Donor or recipient mice were daily treated (5–10 days) with IL-2 at low (25000 IU) or high dose (250000 IU). Immune reconstitution and T cell activation were assessed by *in vivo* bioluminescence imaging and flow cytometry.

Results: IL-2 administration to donor mice resulted in a dose-dependent expansion of Treg in the graft but had no effect on aGVHD development. Treatment of grafted mice with IL-2 beginning on the day of grafting or in a curative schedule did not lead to aGVHD control and higher IL-2 doses accelerated mortality. Mechanistically, IL-2 administration to recipient mice activated all donor T cell subsets, including protective Treg and pathogenic conventional alloreactive T cells. Remarkably, IL-2 induced a dramatic and sustained increased expression of CD25 on donor conventional $CD4^+$ and $CD8^+$ T cells, endowing them with a higher capacity to respond to IL-2 and possibly explaining the loss of selectivity of IL-2 effect on Treg. This loss of IL-2 specificity for Treg was independent of the conditioning of the recipients as it was confirmed in mouse models of GVHD without irradiation and/or lymphopenia.

Conclusions: IL-2 effects can be radically different depending on the immune pathology (T1D, aGVHD), and IL-2 use during aGVHD should be reconsidered.

P1817

Early and gut aGvHD characterize with lowering of blood levels of IFN-gamma and IL-17 producing lymphocytes

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Purpose/Objective: Alloreactivity is a major challenge post HSCT.

Materials and methods: The cytoplasmic expressions of IL-17, FoxP3 and IFN-γ were studied in stimulated PBMC (BFA, Ionomycin and PMA) of alloHSCT pts (83 pts, median age: 41 years, range from 1 to 64 years), 13 pts manifested aGvHD at the time of hematological recovery (early) and in 18 pts with aGvHD was clinically apparent later time post HSCT (late from 26 to 93 days, median 34 days). The cells were labeled for CD4 and intracellulary for IFN-γ (BD, CA), IL-17A and FoxP3 (e-biosciences, CA) detection.

Results: (1) IL-17 ± cells contribution to blood CD4 ± lymphocyte pool: pts with early aGvHD had lower values of IL-17+ cells (median 0.15% versus 0.47%, $P = 0.040$, Mann-Whitney U test), similarly but only tendency was seen in pts developing skin aGvHD later time post HSCT (0.20% versus 0.47%, $P = 0.087$). Gut aGvHD pts behaved rather differently as sharp lowering of IL-17+ cells was seen just at the time of clinical manifestation (0.45% versus 0.20%, $P = 0.063$, Wilcoxon matched pairs test).

(2) IFN-γ ± cells contribution to blood CD4 ± lymph. Pool: tended to be low in pts with clinically apparent early aGvHD (0.09% versus 0.68%, $P = 0.06$), later manifested aGvHD pts had low values only when gut was involved (0.88%,1.12% versus 0.15%, for lacking, having skin and gut aGvHD, respectively, $P = 0.033$).

(3) FoxP3 ± cells contribution to blood CD4 ± lymph. pool: pts prior to overt onset of aGvHD had rather lower values from those seen in cases with overt manifestation of the disease irrespective whether only skin or also gut were involved. However the differences only for skin aGvHD pairs were statistically significant (1.88% versus 6.87%, $P = 0.037$). Notably, contributions of IFN-γ and FoxP3 cells to CD4+ lymph in aGvHD pts tended to be correlated ($R = 0.385$, $P = 0.063$) what was not seen when IL-17+ cells were analyzed.

Conclusions: Both IFN-γ and IL-17 producing lymphocytes levels, being low in blood of the affected cases, mirror aGvHD activity. This is most striking in early aGvHD and in cases with gut manifestation. Low FoxP3 lymphocytes may herald aGvHD then they increase at the time of clinically apparent alloreactivity. FoxP3 lymphocytes take a part in the network rather with IFN-g but not IL-17 producing lymphocytes. Immunologically active lymphocytes likely marginalize in affected tissues.

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P1818

Effect of cord blood regulatory T cells on natural killer cell differentiation and function

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Purpose/Objective: Regulatory T cells (Tregs) may offer a promising treatment for graft versus host disease, the main complication of haematopoietic stem cell transplantation (HSCT). To assess the feasibility of this therapy, it is important to consider their interaction with natural killer (NK) cells, which are described as key effector cells of graft versus leukaemia (GvL). Several groups have studied NK cell suppression by Tregs. However, a better understanding of this interaction using cord blood (CB) as a cell source is necessary, since CB is being increasingly used as a source of stem cells for HSCT.
Materials and methods: This study examined CB cells as a model of transplantation and focused on the effect of Tregs on (1) NK cell phenotype and cytotoxicity and (2) NK cell differentiation from $CD34^+$ stem cells to mature NK cells during a 5-week culture. Phenotype and viability (7AAD-Annexin V) were assessed by flow cytometry and NK cell killing capacity by ^{51}Cr-release assay. To assess Treg effects on different stages of differentiation, Tregs were added at 1:4 ratio with NK cells at 2, 9, 16, 23 and 30 days of culture.
Results: When Tregs were added at day 16, there was a significant decrease in the percentage of committed iNK cells (median, 27.16% NK cells v. 6.24% NK cells with Tregs, $P < 0.01$) and $CD56^{bright}$ NK cells (median, 29.05% NK cells v. 4.78% NK cells with Tregs, $P < 0.05$); yet NK cell killing capacity was not affected ($P > 0.05$). Similarly, whilst isolated NK cells co-cultured with Tregs decreased the expression of activating receptors (CD16, NKG2D, NKp46 and DNAM-1), this effect was not maintained.
Conclusions: So far, no persistent effect of Tregs on NK cell differentiation and function has been detected; therefore, a complete study with activated Tregs is required. This study may help to determine if NK cell-mediated GvL is adversely impacted by CB Treg therapy.

P1819

Embryonic and induced pluripotent stem cell lines are deficient in the processing of minor histocompatibility antigens

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Purpose/Objective: Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) might be useful to generate cells for new transplantation therapies to treat, e.g. heart failure or Parkinson's disease. Among the major problems of these transplantations are the risks of immune rejection and teratoma formation. Therefore, the susceptibility of stem cells and their derivatives to immune rejection mechanisms has to be determined. We have previously shown that mouse stem cells can be killed by activated CTL despite expressing only trace amounts of MHC class I molecules. Now, we wanted to assess the capability of stem cells to process endogenous proteins that function as minor histocompatibility antigens and to become targets of alloreactive CTL.
Materials and methods: ESCs, iPSCs, and as control RMA lymphoma cells (all $H2^b$) were transfected with Ovalbumin (OVA) expression constructs and used in ^{51}Cr release assays as targets for CTL derived from TCR transgenic OT-I mice or alloreactive CTL obtained by immunisation of wild type mice. Expression of MHC class I molecules and major components of the antigen processing machinery was determined by qRT-PCR or flow cytometry.
Results: OVA expressing ESC and iPSC lines were not killed by CTL from OT-I mice in contrast to OVA expressing RMA cells. Stem cells pulsed with the SIINFEKL peptide were killed although less efficiently than RMA cells. Alloreactive CTL also killed the stem cell lines and IFN-γ treatment further increased the lysis although the MHC class I expression was not substantially enhanced as assessed by flow cytometry. Interestingly, the stem cell lines failed to express *TAP2* mRNA in contrast to other genes of the antigen processing machinery.
Conclusions: The data indicate that antigen processing is impaired in ESCs and iPSCs. The very low expression of MHC class I molecules itself is not the limiting factor for recognition and killing by activated CTL. Upon transplantation pluripotent stem cells might become targets for CTL using the direct pathway of allorecognition. However, in allogeneic but MHC-matched transplantations undifferentiated ESCs and iPSCs can be expected to be protected against CTL recognizing minor histocompatibility antigens. This might contribute to the tumour risk of transplants containing undifferentiated stem cells.

P1820

Establishing the role of lymphocytes in hepatic Ischemia reperfusion Injury

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Purpose/Objective: Background: Ischemia reperfusion injury (IRI) is a significant cause of morbidity and mortality (MI, stroke, acute renal failure). Furthermore, it limits the resectability of tumours in liver surgery and the availability of organs for transplantation. Evidence from other groups in a variety of IRI models suggests that IRI is a CD4+ T cell dependent phenomenon.
Aim: Assess the role of lymphocytes in a warm model of hepatic IRI.
Materials and methods: Using a surgical murine model of warm hepatic IRI, the extent of injury was measured using biochemical (ALT) and histopathological parameters to assess liver injury. A number of transgenic mice (RAG1-/-, IL-17RA-/-, OT-II) were used to evaluate the effects various immune populations and antigen specificity of effector populations. Anti-asiola GM1 antibody was used to deplete natural killer (NK) cells.
Results: RAG1-/- mice were protected from IRI, implying that T or B cells are important in the pathogenesis of IRI. Flow cytometric data showed a profound and rapid influx of neutrophils into the site of injury, which appears to be independent of IL-17. There was a relative enrichment of regulatory T cells following reperfusion. Depletion of NK cells was not protective. Injury was not reduced using transgenic mice with limited a repertoire of TCR on conventional T cells, suggesting this is an antigen independent phenomenon.

Conclusions: IRI appears to be dependent on lymphocytes in an antigen independent manner. Recruitment of neutrophils into the site of injury is not IL-17 dependent.

P1821

Evaluation of the use of an immortalized human dermal microvascular cell line as an *in vitro* model to monitor post-transplant endothelial complications

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Purpose/Objective: A number of syndromes have been identified which occur after allogeneic hematopoietic stem cell transplantation (HSCT), including Graft-versus-Host Disease (GVHD), affecting many organ systems. These syndromes are believed to be directly related to microvascular endothelial activation and damage, causing elevated rates of morbidity and mortality. Moreover, anti-endothelial immune responses have not yet been well defined. This study aimed to investigate the use of an immortalized human dermal microvascular endothelial cell line, namely HMEC-1, as an *in vitro* model to effectively monitor and predict post-transplant endothelium-related complications.
Materials and methods: Endothelial cell characteristics of HMEC-1 and their responses to proinflammatory stimulation were evaluated by determining the surface and intracellular expression of molecules common to endothelial cells, employing immunofluorescent stainings and flow cytometry. The stimulation of allogeneic T cell proliferation and allocytotoxicity was analyzed by flow cytometry and the standard 4-h ^{51}Cr release assays, respectively. The immunophenotypic properties of T cells were investigated by determining the flow cytometric surface molecule expression as well as the evaluation of cytokines using commercially available ELISA kits.
Results: This study confirmed that the immortalized HMEC-1 cell line carried most of the characteristics of human microvascular endothelial cells. They responded to proinflammatory stimulation with tumor necrosis factor α, interleukin-1β and interferon-γ by increasing the surface expression of adhesion and class-I MHC molecules. HMEC-1 cells under continuous, uni-directional, non-laminar flow responded similar to HMEC-1 cells under *in vitro* conditions. These cells efficiently stimulated the proliferation of allogeneic T-cells and class-I MHC-restricted allocytotoxic responses. Immunophenotypic analyses of activated $CD4^+$ and $CD8^+$ T cells revealed that they carried the properties of activated $T_{HELPER}1$ and $T_{CYTOTOXIC}1$ lymphocytes, respectively.
Conclusions: The generation of responses similar to primary endothelial cells by HMEC-1 cells after proinflammatory stimulation and their apparent antigen presenting capacity suggest that HMEC-1 cells might be an appropriate option for individual patient-based *in vitro* monitoring of post-transplant complications, using either patient derived cells or sera.

P1822

Gaining an advantage: mycophenolate-resistance for adoptively transferred cells

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Purpose/Objective;Post-transplant lymphoproliferative disorder and viral infection with CMV are attractive targets for use of adoptive immunotherapy. In the post-transplant period, continued treatment with immunosuppressive drugs to prevent rejection or graft versus host disease suppresses adoptively transferred cells. A T333I, S351Y mutant inosine-5'-monophosphate dehydrogenase (mIMPDH2), confers 2400-fold resistance to MMF. We aim to transduce CD8 T cells with both a TCR specific for CMV or EBV antigens in addition to mIMPDH2 to enable treatment of infection or post-transplant lymphoproliferative disorder (PTLD) during ongoing mycophenolate mofetil (MMF) therapy.
Materials and methods: Cells were retrovirally transduced using SFG based vectors. To track transduced cells, the mIMPDH2 gene is fused with eGFP. Either a hypofunctional (C331A) IMPDH2 mutant fused to eGFP or eGFP alone were used as controls. Both murine MACS sorted CD8 T cells and a thymoma cell-line (BW 5147) were used for *in vitro* study. Murine CD8 cells were either restimulated with cognate peptide or cultured with media containing interleukins 2, 7 and 15. The active metabolite of MMF, mycophenolic acid (MPA), was used *in vitro*.
Results: Murine CD8 T cells transduced with mIMPDH2 exhibit a selective advantage compared to control transduced cells when exposed to MPA *in vitro*. Enrichment of transduced cells is most marked following restimulation of mIMPDH2 transduced OT1 transgenic T cells with cognate peptide, using ratios of %GFP positive in MPA versus no MPA to correct for different levels of transduction, mIMPDH2 = 7.12, eGFP = 0.77 (two-tailed paired t-test $P = 0.002$). Selection is also seen in an antigen-independent fashion during culture in the presence of IL-2, IL-7 and IL-15 (mIMPDH2 = 4.37; eGFP = 1.10 $P = 0.018$). Cells transduced with mIMPDH2 exhibit less apoptosis (Ratio %annexin V positive MPA:No MPA, mIMPDH2 = 1.16; C331A = 2.93 $P = 0.0101$) and are able to overcome the MMF-induced cell cycle arrest in G1 phase, when compared to non-transduced cells.
Conclusions: *In vitro* experiments have confirmed the resistance phenotype conferred by mIMPDH2. Work is ongoing using *in vivo* murine models of PTLD and viral antigen challenge to assess the function of adoptively transferred cells co-transduced with TCR and mIMPDH2 during ongoing MMF therapy.

P1823

HLA-DR expressing endothelial cell expansion of allogeneic $CD4+$ T towards either pro-inflammatory Th17 or suppressive Treg is regulated by HLA-DR mediated signaling

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Purpose/objective: The major obstacle to organ transplantation is chronic rejection due to the persistence of the alloimmune response due to Major Histocompatibility Complex molecule (MHC) expression on donor cells. Allograft microvascular endothelial cells (ECs) express both MHC class I and class II molecules and therefore have a dual role as both stimulators of, and targets for the allo-immune response. We have recently shown that ECs can generate either regulatory $CD4^+$ T cells (Tregs) or pro-inflammatory $CD4^+IL\text{-}17^+$cells (Th17) but the mechanisms regulating the balance between Tregs and Th17 remain to be determined.
Materials and methods: We have established a model of either inducible or constitutive HLA-DR expression in microvascular endothelial cells using lentiviral vector infection. This model was used to study (i) the mechanisms of EC induced activation of allogeneic CD4+ T cells and (ii) the outcome of HLA-DR antibody pre-activation of

endothelial cells on their ability to polarize towards the Treg or Th17 lineages.

Results: Under inflammatory conditions, HLA-DR expression of human microvascular ECs allows them to orient the $CD4^+$ T lymphocyte response towards either a Th17 pro-inflammatory response or towards a regulatory T cell response. Expansion of Th17 depended upon IL-6 secretion by endothelial cells which led to STAT-3 activation in allogeneic $CD4^+$ T cells associated with their proliferation, while Treg expansion was dependent upon their interaction with endothelial cells mediated by the adhesion molecule ICAM-1 (CD54). Pre-treatment of ECs with HLA-DR antibody induced increased secretion of IL-6 leading to increased expansion of alloreactive Th17 cells.

Conclusions: These data reveal that firstly the human microvascular EC can regulate polarization of the memory $CD4^+$-T cell response towards either cytokine-dependent inflammation or contact-dependent regulation and secondly that HLA-DR antibody pre-activation of EC increases their ability to induce a pro-inflammatory Th17 response. Together these results provide evidence for a link between the role of the EC as a target of HLA-DR antibody binding and as an allostimulator of the pro-inflammatory Th17 response. These data may contribute to our understanding of complement independent mechanisms of endothelial cell damage in the presence of HLA class II alloantibodies.

P1824

IL-10 haplotypes associated with infection susceptibility in autologous bone marrow transplantation in multiple myeloma patients

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Purpose/Objective: Bacterial, viral and fungal infections are major obstacles to the success of onco-hematological patients undergoing stem cell transplantation (SCT). SCT increases patient's vulnerability to infections due to immunological changes related to the conditioning regimen. The introduction of Autologous Stem Cell Transplantation (ASCT) increased the overall survival of these patients, but infection still remains an important complication of the procedure. The increased expression levels of cytokines have an important relationship with the immune response. Single Nucleotide Polymorphisms (SNPs) in the promoter region of cytokine genes are responsible for altering the levels of expression, thereby affecting the immune response. Therefore, we hypothesized that SNPs in the IL-10 gene, as well as the haplotypes formed by them, are associated with the susceptibility to infection in multiple myeloma patients submitted to ASCT.

Materials and methods: We have clinically assessed the infection status of the patients for the following conditions: fever of unknown origin, death during the neutropenia phase, *fungous* blood stream infection (F.BSI), superinfection, bacteremia (Gram-positive and Gram-negative), invasive *fungous* infection. Genomic DNA was extracted from mobilized peripheral-blood stem cells of 148 patients from the Bone Marrow Transplantation unit of Federal University of Rio de Janeiro. Genotyping was carried out for the IL-10 SNPs A-1082G, A-592C and T-819C using the Real-time PCR assays. Haplotype frequencies were estimated with Haplo.stats® and all the other statistical analysis were performed with SPSS®.

Results: We have not observed deviations from the Hardy-Weinberg equilibrium for the genotypes. The SNP A-1082G was associated with superinfection ($P = 0.020$). Interestingly, the ATA haplotype was also associated with superinfection ($P = 0.016$). In addition, the ATA haplotype was associated with F.BSI ($P = 0.004$). Finally, the ACC haplotype was associated with Gram-negative bacteremia ($P = 0.040$) and superinfection ($P = 0.048$).

Conclusions: The results showed that despite the primary predisposition to infection typical of multiple myeloma, the presence of the variants studied significantly affected the susceptibility to serious infections and outcomes of ASCT.

P1826

Immunological markers in early prognostic of cardiac allograft vasculopathy (CAV) on heart transplantation

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Purpose/Objective: Cardiac allograft vasculopathy is a diffuse form of coronary atherosclerosis on transplanted heart (TH), progresses to chronic rejection and is one of the major causes of graft failure. Although it is a multifactor process, the recipient immunologic response against allogeneic graft is the major factor of this endothelial dysfunction. Since the TH is denervated, CAV is a silent phenomenon and it's frequently diagnosed at an advanced stage of the disease. There is a need to develop early, non-invasive and accessible diagnostic techniques.

Allospecific Th1 lymphocytes have been described as effector cells in CAV development. In a previous study, we reported the usefulness of the Th1/Treg ratio as a prognostic marker of CAV. The objective of this study is to identify early changes in effector $CD4^+$ $CD45RO^+CD25^{-/low}CD127^{hi}$ (Tact) and regulatory $CD4^+CD45RO^+$ $CD25^{hi}CD127^-$ (Treg) lymphocyte subsets on peripheral blood that correlate with CAV development.

Materials and methods: Thirty-two heart transplanted (HTx) patients were studied during 2 years follow-up at Hospital de Sant Pau. In addition 24 long-term patients (14 CAV-diagnosed, 10 no CAV) and 12 blood bank volunteers were studied as the HTx and healthy control groups respectively.

Flow cytometry analysis of $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, $CD56^+$, Th1, Tactand Treg from periodically obtained peripheral blood lymphocytes were studied. Endomyocardial biopsies and coronariography were conducted to complete the global study.

Results: A progressive increase in the Th1 and Tact subsets and Th1/Treg and Tact/Treg ratios was detected during follow-up. Patients with CAV displayed a significant Tact/Treg increase versus long-term patients without CAV ($P < 0.0002$). Significance of the Tact values was $P = 0.03$.

Tact/Treg ROC analyses showed an area under de curve AUC of 0.962, the estimate sensitivity was 92.3%, specificity, 90.0%, the positive prediction value PPV, 92.9%, and the negative prediction value NPV, 100%. Th1/Treg ROC results were AUC 0.90, sensitivity 91.6%, specificity 90.0%, PPV 92.3% and NPV 100%.

Conclusions: These results indicate that the Tact/Treg ratio may represent a valuable marker to monitor allospecific T cell responses in peripheral blood and in addition changes in Tact/Treg ratio may help in the early detection of patients at risk of CAV.

P1827

Impact of urinary tract infections and CMV infections on renal graft function: is there anything to do for an immunologist?

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Purpose/Objective: The current research in transplantology, focused mostly on rejection mechanisms, corresponds with last years' discoveries at autoimmunity which refers to the place of Th17 cells in inflammation and recurrent infections. However little is known about transplant immunity in the context of recurrent infections according to basic as well as translational studies. The aim of the following study was first to analyze the impact of urinary tract infections (UTI) and cytomegalovirus infections (CMV) on long term- renal graft function, second to compare the demographic profile of group with impaired graft function in terms of infections with the patients suffering from autoimmune diseases.

Materials and methods: Group of 159 renal transplant recipients was analyzed. The subgroup with at least one confirmed UTI or CMV episode was differentiated with age, sex and the number of episodes. Creatinine level during the first 24 months post-transplant was followed. Trends in creatinine level as well as glomerular filtration rate (GFR) 24 months post-transplant in groups with and without UTI and CMV were analyzed. Regarding normal distribution displayed in every subgroup, the average GFR rates were compared with t *Student test.

Results: The total incidence of 41% was observed and the total number of 171 of infectious episodes was diagnosed, including 116 UTI episodes (68%) and 18 CMV episodes (11%). The increasing creatinine level was observed in group with at least one UTI or CMV episode which was positively correlated to the trend in patients with recurrent UTIs and negatively to the trend in patients without infections. Increasing creatinine level was observed in recipients with at least one UTI episode, younger than average for the analyzed group (<43) comparing to the older recipients (>44) with at least one UTI episode. Stable creatinine level was observed in women with at least one UTI episode by the opposite to decreasing creatinine level in women without infections. Lower GFR scores 24 months post-transplant were observed in patients with recurrent infections and women with at least one UTI/CMV episode comparing to the patients without infections in general as well as to men and women separately.

Conclusions: Recurrent UTIs and CMV infections negatively influence long-term renal graft function. The profile of patients with impaired graft function (women and younger patients) resembles the epidemiology of autoimmune diseases. The role of recurrent UTIs in this phenomenon points the need of further immunological studies on this area.

P1828

Importance of TNF-alpha and but not by of interleukin 17 in allogeneic hematopoietic stem cell transplantation (HSCT)

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Purpose/Objective: The allogeneic hematopoietic stem cell transplantation HSCT is a technique that can restore bone marrow function; the main complications are development of graft versus host disease (GVHD), relapse and graft loss. Among the factors that influence the success of allogeneic HSCT include the good reconstitution and correct control of immune response to prevent the development of GVHD which is a response triggered by recognition of alloantigens and produces an immune response characterized by an inflammatory reaction in which cytokines play an important role. Determinate the Th1, Th2 and Th17 cytokine profile and TNFα in patients with hematological diseases after HSCT and correlate them with the development and severity of GVHD.

Materials and methods: We analyze samples of patients with hematologic malignancies undergoing HSCT submitted to the Unidad M*dica de Alta Especialidad, Centro M*dico Nacional La Raza, IMSS. Samples were taken prior to the HSCT, and at 30, 60, 100 and 180 days after transplantation. The following cytokines were quantified by flow cytometry: IFNγ, IL10, IL17A, IL17F in CD4+ cells, IFNγ in CD8+ cells and TNFα in CD14+ cells; And by CBA: IFNγ, IL2, IL10, IL4, IL17A, and TNFα.

Results: We studied 7 patients were included and divided, into two groups: The first group patients with GVHD and the second group patients without GVHD. Seven healthy donors were included as controls. Also we analyzed a patient with syngeneic transplant, who was used as alternative control.

In the first group, it was observed an increase of TNFα without co-stimulation. In cell cultures showed an increase in of the Th1 cytokine profile as IFNγ and IL2. This correlated with the onset and severity of GVHD.

In the second group, it was observed a lack of production of cytokines, such as basal in the presence of co-stimulation. This pattern was observed correlated with graft loss, infections with opportunistic microorganisms and relapse. Unlike other reports in murine models, no significant changes in the Th17 cytokine profile.

Conclusions: High levels of TNFα play an important role in the development and severity of GVHD, but not in the maintenance of the graft. We didn't observe that an apparent role of Th17 profile play an important role in the development or severity of GVHD. In contrast, the fact that the patients' cells were capable to respond to polyclonal stimuli correlates with graft success.

P1829

In vitro-generated myeloid-derived suppressor cells inhibit graft-versus-host disease in a MHC class I mismatched bone marrow transplantation model

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Purpose/Objective: Bone marrow transplantation (BMT) is a curative treatment modality for hematologic malignancies, since alloantigen specific donor T cells in the graft eliminate residual tumor cells (graft-versus-tumor effect: GVT). However, these T cells are also responsible for the induction of graft-versus-host disease (GVHD) by attacking alloantigen expressing recipient tissue, leading to significant morbidity and mortality. Therapeutic goal in allogeneic BMT is the inhibition of GVHD induction while maintaining the GVT effect. Myeloid-derived suppressor cells (MDSCs) represent a population of myeloid precursors able to suppress T cell activation and proliferation and were therefore tested for their ability to prevent GVHD after allogeneic BMT.

Materials and methods: BM cells were cultured in the presence of GM-CSF and G-CSF to generate MDSCs. To test, whether MDSCs inhibit GVHD induction, B6-derived MDSCs were co-injected at different concentration with B6-derived allogeneic BM and spleen cells (SC) into lethally irradiated B6.bm1 mice and weight loss, GVHD score and survival of transplanted mice were determined. Immunosuppressive effects of MDSCs were defined by analyzing donor chimerism, allogeneic T cell activation and homing and survival of MDSCs.

Results: Culturing BM cells in the presence of GM-CSF and G-CSF induced in 90% of the cells a $CD11b^{+}Gr\text{-}1^{+}$ MDSC phenotype. These MDSCs effectively suppressed allogeneic T cell proliferation *in vitro*. Co-transplantation of *in vitro*-generated MDSCs together with allogeneic BM and SC inhibited GVHD in a dose-dependent manner. A single injection of $1*10^{7}$ MDSCs totally protected from GVHD-induced death. Transplanted MDSCs were detected in the blood, spleen, liver and bone marrow of transplanted mice throughout the whole experiment. However, transplanted MDSCs did not interfere with phenotype and activation of allogeneic T cells and did not affect donor chimerism.

Conclusions: In summary, we could show that transplantation of *in vitro*-generated MDSCs efficiently prevented clinical GVHD, suggesting that MDSCs might be a useful cellular GVHD-therapy after allogeneic BMT. Defining their mechanisms of immunosuppression and their effect on T cell mediated tumorcytotoxicity will further elucidate whether usage of MDSCs might be applicable in the clinic.

P1830

Induction of immunosuppressive MDSC by extracorporeal photopheresis in patients with chronic GvHD

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Purpose/Objective: Chronic Graft-versus-Host-Disease (cGvHD) remains the main non-relapse related cause of death in allogeneic hematopoietic stem cell transplantation. In general, treatment of cGvHD is based on administration of immunosuppressive drugs. However, extracorporeal photopheresis (ECP) has become a well-accepted treatment for cGvHD. Induction of regulatory T cells (Treg) by ECP seems to be important against cGvHD, though the mechanisms of Treg differentiation still remain enigmic. Recently, $CD14^{+}HLA\text{-}DR^{low}$ monocytic myeloid derived suppressor cells (moMDSC) were found to support Treg differentiation in humans. Therefore, we hypothesize that ECP induces moMDSC which in turn facilitates Treg differentiation in patients with cGvHD.

Materials and methods: cGvHD was diagnosed according to NIH criteria and confirmed by skin biopsy. Two consecutive cycles of ECP were performed per week for 12 weeks. Blood was obtained after informed consent from patients with cGvHD and from age and gender matched healthy donors (HD). After peripheral blood mononuclear cells (PBMC) isolation by Ficoll, Treg and moMDSC frequencies were determined by flow cytometry. MoMDSC function was tested in autologous CFSE-based-proliferation assays. In addition, the ability moMDSC to drive differentiation of Treg was tested in autologous co-cultures of moMDSC and $CD4^{+}$ T-cells.

Results: Patients ($n = 4$) with cGvHD showed lower Treg and moMDSC frequencies as compared to healthy donors ($n = 6$) ($P < 0.05$). Serial measurements performed at week 4, 8 and 12 after starting ECP treatment showed a significant increase of moMDSC and Treg frequencies in three out of four patients ($P < 0.05$). *In vitro*, moMDSC inhibited polyclonal T cell proliferation and were found to induce T cells with the phenotype of Treg.

Conclusions: Our preliminary data indicate that ECP increases the frequency of $CD14^{+}HLA\text{-}DR^{low}$ moMDSC in patients with cGvHD. *In vitro*, moMDSC suppress T-cell proliferation and support Treg differentiation. Thus, moMDSC induction by ECP might be of great therapeutic relevance in patients with cGvHD.

P1831

Interleukin-7 receptor polymorphisms: Impact on regulatory T cells in patients undergoing myeloablative allogeneic stem cell transplantation (SCT)

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Purpose/Objective: Interleukin-7 (IL-7) is a cytokine essential for T cell development in the thymus and maintenance of the peripheral T cell population. The receptor (CD127) is highly expressed in most naïve T-cells, but is expressed at low levels in FoxP3+ T regulatory cells. The CD127 chain is shared with the receptor of Thymus Stromal LymphoPoietin, a cytokine with a key role in the development of natural FoxP3+ T cells in the thymus in Hassal's corpuscles and in the induction dendritic cells with low IL-12p40 release, that drive the generation of induced FoxP3+ T cells. We have identified a number of single nucleotide polymorphisms in the exons of the gene encoding IL-7Rα, including rs1494558 C/T and rs1494555A/G, and found that the minor alleles are associated with acute graft versus host disease (aGvHD) and mortality in patients undergoing SCT. We hypothesized that the generation of regulatory T cells in SCT patients is influenced by IL-7Rα genotypes of the donor.

Materials and methods: Blood CD4+ T cells were isolated using immunomagnetic beads and mRNA profiles related to regulatory functions (FoxP3, CD25 and CTLA-4) were measured by Taqman semi quantitative RT-PCR. mRNA profiles were expressed as ΔCt using CD4 as a reference gene. IL-7R SNPs were determined by a sequence specific PCR system. Two study populations were included: 1) 155 healthy individuals aged 33.5 years (4.7;62) and 2) 92 SCT patients aged 23.1 years (1.8;53.0). The latter were investigated 1 year after the transplant, and T cell phenotypes were analyzed for associations with donor IL-7R genotypes.

Results: In SCT, rs1494555 genotypes of the donor were significantly associated with CTLA-4 mRNA levels in CD4+ T cells (AA ΔCt: 1.2 (-4.0;3.9) (median, ranges) ($n = 46$), AG/GG: 2.0 (-0.8;3.6) $P = 0.012$). For FoxP3 a borderline significant association was seen (AA: 1.0 (0.8;1.5), AG/GG: 1.2 (-0.4;3.9), $P = 0.075$), while this SNP was unassociated with CD25 mRNA levels ($P = 0.21$). A similar pattern was observed for the closely linked SNP rs1494558. No significant associations were found in healthy donors.

Conclusions: These data indicate that the genotypes previously associated with aGvHD, are related to reduced levels of regulatory T cells, thus providing a biological mechanism for the previous clinical associations. In contrast, IL-7Rα genotypes are not associated with the level of regulatory T cells in healthy adults, suggesting a differential role of genetic variations in IL-7Rα, depending on whether the lymphocyte population is in a steady state or in a regenerative phase.

P1832

Intravenous immunoglobulin selectively enhances CD4+CD25+FoxP3+ Tregs function in patients

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Purpose/Objective: Intravenous immunoglobulins (IVIg) are used as anti-inflammatory therapy for autoimmune diseases. We and others have shown that IVIg treatment also protects against acute rejection in liver transplant patients. In mice we observed that prevention of allogeneic skin allograft rejection by IVIg is mediated by $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs). To determine whether IVIg stimulates Treg in humans *in vivo*, we studied Treg activation and suppressive function in patients treated with IVIg.

Materials and methods: We included 26 patients who where treated with IVIg monotherapy because of hypogammaglobulinaemia or autoimmune diseases. Blood was collected before, immediately after, and 7 days after IVIg infusion. Changes in Treg markers, serum cytokine and IgG levels were measured by flowcytometry and ELISA. The suppressive capacity of $CD4^+CD25^+CD127^-FoxP3^+$ Treg was studied *ex vivo* by co-culturing purified Treg with conventional $CD4^+CD25^-CD127^+$ T-helper cells (Tconv) from the patients stimulated with phytohemagglutinin (PHA). To study the mechanism of Treg activation *in vitro*, purified $CD4^+CD25^+CD127^-$ were cultured with IVIg and activation markers were measured by flowcytometry.

Results: Serum IgG levels increased 2.2-fold directly after IVIg treatment ($P < 0.001$). IVIg treatment selectively activated $CD4^+CD25^+$ $FoxP3^+$ Tregs, as revealed by enhanced HLA-DR expression ($T = 0$ to $T = 7$: +31%, $P < 0.01$), while this remained unchanged on conventional $CD4^+$ T cells. In addition, FoxP3 expression increased significantly after treatment ($T = 0$ to $T = 1$: +23%; $P < 0.01$ and $T = 0$ to $T = 7$: +30%; $P < 0.01$). Serum concentration of the anti-inflammatory cytokine IL-10 increased 2.5-fold shortly after treatment ($P = 0.02$). In *ex vivo* suppression assays, the Treg suppressive capacity increased 2.2-fold after IVIg treatment ($P = 0.02$). *In vitro*, we observed that Tregs internalized IVIg rapidly, which was followed by upregulation of the activation marker CD69.

Conclusions: We show that IVIg, which has been proven to be a safe immunosuppressive agent, selectively activates and enhances the suppressive capacity of Tregs in humans *in vivo*. Since proper activation of Treg protect against allograft rejection, IVIg may be introduced as a safe alternative immunosuppressive treatment after organ transplantation.

P1833

Lymphocyte subpopulations recovery post alloHSCT and herpes viruses reactivation influenced themselves in a mutual fashion

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Purpose/Objective: Immunological recovery post alloHSCT is influenced by the conditioning regimen, cellular profile of transplant material and further post transplant is shaped by environmental factors. CMV, EBV and HHV-6 predominately infect lymphoid cells and also influence thymic and bone marrow microenvironment.

Materials and methods: We followed 44 patients (age 1 to 64 median 45 years, 21 ric/mac, 21/23, sib/mud 21/23, BM/PBPC 4/40) for the pace of lymphocyte recovery at four consecutive time points beginning when the number of lymphocytes in blood exceeded 200 cells/μl after transplantation (median day 35 range 31−53). We used the method of flow cytometry to determine lymphocytes of CD4(+), CD8(+), CD20(+) profile and CD4+ T cell subsets: Central memory (CM) CD4(+) CCR7+CD45- and Effector memory (EM) CD4+CCR7-CD45+. CMV, EBV and HHV-6 copies were measured in blood with the use of QT-PCR.

Results: It appears that: Herpes viruses reactivations were seen in 27 (61%) patients, HHV-6 in 11 patients, EBV 15 and CMV in four patients. HHV-6 reactivation occured rather early after transplantation and was followed by reactivation in eight cases.

At the beginning of immune recovery CD4+ lymphocyte and their EM subset were lower in patient with CMV copies when compared to those without them (CD4+ cells 44/μl versus 120/μl $P = 0.066$ Mann Whitney U test) (CD4+ EM cells 25/μl versus 75/μl $P = 0.088$ Mann Whitney U test) .

Three weeks later the presence of HHV-6 was associated with a decrease in number of CD4+ cells, CD4+ CM and CD4+ EM cells for patients with HHV-6 copies when compared to group without HHV-6 copies, which was independent of aGvHD manifestation. (CD4+ cells 93/μl versus 190/μl; $P = 0.044$ Mann Whitney U test); (EM cells 41/μl versus 142/μl; $P = 0.015$ Mann Whitney U test) (CM cells 6/μl versus 18/μl; $P = 0.015$ Mann Whitney U test).

All CMV infected patients survived and showed an increase in CD8(+) lymphocytes in comparison with those lacking CMV infection 5−6 weeks after transplant. (182 k-k/μl versus 535 k-k/μl $P = 0.017$ Mann Whitney U test).

Patients having EBV copies had higher proportion of CD20(+) cells when compared to those lacking EBV copies (19/μl versus 9/μl; $P = 0.077$ Mann Whitney U test).

Conclusions: Low lymphocyte CD4(+) and CD4+ EM count precedes CMV and results from HHV-6 infection. HHV-6 infection leads to a decrease of lymphocyte CD4+ CM count.

P1834

Mixed chimerism after allogeneic stem cell transplantation for severe aplastic anemia

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Purpose/Objective: Aplastic anemia (AA) is an autoimmune disorder involving the hematopoietic stem cell progenitors (HSCP) in the bone marrow (BM). As a result, the lymphocyte mediated attack to HSCP produces a BM failure, with a variable degree of cytopenias in the peripheral blood cell counts.

Donor«s chimerism after allogeneic stem cell transplantation (Alo-SCT) measures the proportion of blood cells provided by the donor's HSCP in contrast with the ones coming from the recipient. Patients with increased or prolonged mixed chimerism (MC) are at high risk for graft failure and relapse of the disease. The value of MC after Alo-SCT for non malignant disorders as AA is less established.

We present two cases of AA that underwent Alo-SCT from a full matched sibling donor, after the same conditioning regimen, graft versus host disease (GVHD) prophylaxis and stem cell source. Both patients achieved a complete response of the AA with normalization of their peripheral blood counts despite an opposite evolution of their chimerism analysis monitoring.

Materials and methods: Stem cell source G-CSF 10 μg/kg per 5 days, and BM harvest (mobilized BM).

1 Conditioning regimen: cyclofosfamide 200 mg/kg (total dose) and ATG 6 mg/kg (total dose)
2 GVHD prophylaxis: methotrexate (d +1, +3, +6, +11) and cyclosporine from day-2 onwards.
3 Patient A: a 36 years old man who underwent Alo-SCT from his 33 years old brother donor.
4 Patient B: a 53 years old woman who underwent Alo-SCT from her 48 years old sister donor.
5 Chimerism assay was made analyzing Short Tandem Repeats in two cells populations in peripheral blood: total mononuclear cells (TMC) and T lymphocites (CD3+).

Results: Patient A achieved full donor chimerism at d+213 after cyclosporine reduction. Patient B did not achieve full donor chimerism at d +530 (75% in TMC, 60% in CD3+). In patient B, a progressive increased in MC did not correlate with impairment in blood cell counts.

Conclusions: Chimerism assay after Alo-SCT is a useful tool that measures the kinetics of the engraftment of donor derived blood cells. MC and even the complete conversion to recipient full chimerism and autologous reconstitution of recipient HSCP may occur without the relapse of the AA, showing an immunomodulatory effect of the Alo-SCT beyond its effect of replacing the HSCP from the recipient to the donor«s derived ones.

P1835
NK cells in kidney transplantation

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Purpose/Objective: Following kidney transplantation, the involvement of NK cells in rejection episodes or immunological tolerance is discussed controversially. The peripheral NK cell repertoire of KTx patients at 3 or 6 months post Tx was investigated in parallel to the protocol biopsy for histopathological evaluation of the graft. The alterations were correlated to the biopsy status ('unsuspicious', T cell-mediated rejection TCMR or borderline changes) and immunosuppression, respectively.

Materials and methods: Peripheral blood lymphocytes of KTx patients ($N = 100$) at the time of the protocol biopsy at 3 or 6 month after Tx were isolated after informed consent of the patients. In addition to the 'truecount' quantification of T, B, NK cells (cell/μl), the composition of the NK cell repertoire was determined by flow cytometry for the expression of several NK receptors including KIR and CD94/NKG2. In addition to NK cells, receptor expression was also addressed in T cell subsets. In some cases, protocol biopsies and peripheral blood was available at multiple time points after Tx and, thus, changes over time and immunosuppression (CsA versus Tac versus mTOR inhibitors) could be defined.

Results: The numbers of NK cells/μl blood differed between patients after KTx with 'unsuspicious' grafts and TCMR or borderline histopathology although not at a significant level. However, the distribution of $CD56^{bright}$ versus $CD56^{dim}$ NK cells in these patient cohorts displayed significant differences compared to healthy donors. In addition, the NK subset analyses revealed substantial differences in the numbers of KIR+ NK cells between the patient cohorts.

Conclusions: In our first analyses with patients between 3 and 12 months after KTx, we could demonstrate that the numbers of NK cells in peripheral blood differs between patients with kidney grafts unsuspicious of rejection and biopsy-proven TCMR or borderline changes. In addition, a more detailed NK cell subset analysis revealed significant differences in NK subset distribution between the patient cohorts and also, for some subsets, the immunosuppressive treatment groups mTOR inhibitors, in particular. Therefore, we assume that the composition of peripheral NK cells represents an important hallmark of the immune status, rejection versus tolerance, in kidney transplantation. (This work was funded by the IFB-Tx, DFG TRR77, A3 snd SFB738 B8 projects and the HGF Alliance Immmunotherapy).

P1836
Nuclear factor of activated T cells (NFAT) regulates acute graft versus host disease

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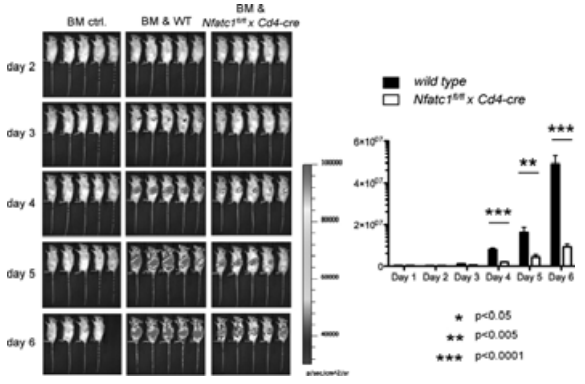
Purpose/Objective: Acute graft versus host disease (aGvHD) is a life-threatening immunological complication after allogenic stem cell transplantation. Patients with ongoing aGvHD are treated with cyclosporin A (CsA) or tacrolimus (FK506), both inhibiting the calcium-calcineurin pathway. Blockade of calcineurin results at first in repression of Nuclear Factor of Activated T cells (NFAT) activation, but may also affect other transcription factors such as NF-κB. Given the side effects of (long term) calcineurin-inhibition we explored the contribution and specific function of individual NFAT-factors in T cells to the overall pathogenic mechanisms in a mouse model of aGvHD.

Materials and methods: We transplanted luciferase-labeled allogeneic T cells from NFATc1- and NFATc2-deficient donor mice along with bone marrow stem cells and monitored the progression of aGvHD by an *in vivo* bioluminescence imaging technique.

Results: Inactivation of both, NFATc1 or NFATc2 resulted in a milder and decelerated clinical course of aGVHD, apparently due to impaired secretion of Th1 cytokines such as IFNγ, TNFα, or IL-2.



Conclusions: We anticipate that more specific therapeutics to block NFAT activation might benefit the clinical treatment of aGvHD by reducing overall side effects, nevertheless facilitating graft versus leukemia/tumor reaction.

P1838

Pentraxin 3: a new potential biomarker for predicting and monitoring graft-versus-host disease (GvHD) in allogeneic hematopoietic stem cell transplantated (HSCT) patients

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Purpose/Objective: GvHD is a major obstacle to safe HSCT. Reliable biomarkers facilitating the monitoring of this invalidating disease are warranted to improve its management. The long pentraxin (PTX) 3 is locally produced at sites of inflammation. Its rapid increase in several inflammatory diseases and the correlation between PTX3 levels and disease severity suggest its potential usage as GvHD marker.

Materials and methods: We collected plasma samples from 71 HSCT pediatric patients, before the beginning of the conditioning regimen and weekly from the transplant day (day 0), until day 100. Thirty-three patients developed acute GvHD within day 28, 21 between day 28 and 100, while 17 never developed it. Concerning GvHD patients, plasma was further collected the day of GvHD onset. PTX3 levels were monitored by ELISA.

Results: To investigate the possible use of PTX3 as GvHD marker, we evaluated its plasma levels at disease onset. Since PTX3 values could be influenced by infections, we compared PTX3 levels at GvHD onset before day 28 after HSCT, time-frame free from infectious complications in our cohort, with time-matched levels of patients who did not develop GvHD within day 28 (no GvHD). The median PTX3 level at GvHD onset was 33 ng/ml (range = 11−847), significantly higher (Wilcoxon P-value<0.0001) than the no GvHD group (median level = 14, range = 4−58 ng/ml). To investigate its predictive potential, we compared PTX3 levels at day 0, 7 and 14, normalized for each patient by his baseline (PTX3 value before conditioning regimen), between patients with early GVHD occurrence (within day 28) and no GVHD patients. We observed that the conditioning regimen induced an increase of PTX3 levels compared to the baseline in both groups, without significant differences. On the contrary, at day 7 and 14 the median PTX3 level were significantly higher in patients experiencing GVHD within day 28 (normalized change = 3.03) compared to the no GVHD group (normalized change = 1.1; Wilcoxon P-value = 0.05).

Conclusions: These results show that PTX3 increases rapidly in patients with acute GvHD, candidating this molecule as an easily measurable soluble factor useful to corroborate the clinical monitoring of the pathology. Furthermore, if confirmed in a larger cohort of patients, PTX3 could represent a sensitive biomarker for predicting patients at high risk for developing GvHD early after HSCT.

P1839

Pretransplant IFNgamma secretion by CMV-specific CD8+ T cells (Quantiferon®-CMV) can predict the risk of cytomegalovirus replication after solid organ transplantation

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Purpose/Objective: We analyzed pretransplant CMV-specific CD8+ T cells secreting IFNγ using the QuantiFERON®-CMV assay to determine whether this assessment provides prognostic value of CMV replication after transplantation.

Materials and methods: This longitudinal study was carried out in two centers of the REIPI network (Reina Sofia University Hospital, Cordoba, and Cruces University Hospital, Bilbao, Spain). Adult patients awaiting lung or kidney transplantation were recruited from March 2009 to June 2010. A total of 55 were evaluable patients. IFNγ production by CMV-specific CD8+ T-cells was assessed pretransplant using the QuantiFERON®-CMV assay. A result was considered positive when the CMV antigen response minus the negative control response was >0.2 IU/ml of IFNγ. CMV load (copies/ml) was monitored for 12 months after transplantation.

Results: All CMV-seronegative recipients were QuantiFERON®-CMV negative [QF_{CMV}(-)], (11/11). However, in CMV-seropositive recipients [R(+)] 30/44 (68.2%) were QF_{CMV}(+) and 14/44 (31.8%) were QF_{CMV}(-). When we analyzed the incidence of post-transplant CMV in these two groups within R(+) recipients we found that 7/14 (50%) of R(+) QF_{CMV}(-) recipients developed CMV replication after transplantation, whereas the virus replicated only in 4/30 (13.3%) of R(+) QF_{CMV}(+) patients. In the kidney transplant recipients group, CMV replicated earlier post-transplant, reached a higher peak CMV load, replication episodes were longer and the frequency of CMV disease was higher in R(+) QF_{CMV}(-) patients than in R(+) QF_{CMV}(+) recipients.

Conclusions: The determination of pretransplant IFNg production using the QuantiFERON®-CMV assay might be useful in predicting the risk of post-transplant CMV replication in solid organ transplantation as it re-classifies patients more accurately than serostatus. R(+) kidney transplant patients with pretransplant QF_{CMV}(-) should, therefore, be managed as high risk patients, by which this pretransplant assay could be used in the clinical setting to guide physicians in applying the adequate anti-CMV therapy after solid organ transplantation.

P1840

Radiation-free conditioning for the establishment of mixed chimerism and allogeneic tolerance in aged mice

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Purpose/Objective: Mixed hematopoietic chimerism is a powerful means of inducing allogeneic tolerance. Clinically, this is a favourable outcome as it allows long-term acceptance of the allograft, eliminates toxicities associated with lifelong immunosuppression and maintains immunocompetence. We aimed to develop a radiation-free protocol to induce tolerance to MHC-mismatched skin allografts and apply this model to an aged setting, combining aged experiments with strategies to boost thymic output.

Materials and methods: Young (6−8 week) or old (12 month) B6.SJL-Ly5.1 (H-2^b) mice received low-dose busulfan on day -4, followed by two doses of T cell-depleting antibodies to CD4 (GK1.5)

and CD8 (2.43) on days -3 and -1. On day 0 mice were transplanted with B10.Br (H-2^k) allogeneic whole BM, T cell-depleted BM or purified lineage negative Sca-1^+ c-Kit^+ (LSK) progenitor cells. Rapamycin was administered at 3 mg/kg/day for 28 days following BMT. Mice received a full-thickness allograft, derived from B10.Br and Balb/c (H-2^d) tail skin at 12 and 24 weeks post BMT respectively. To boost thymic function via sex steroid reduction, aged mice received a 3-month depot administration of Lupron (leuprolide acetate), a luteinising hormone releasing hormone agonist, 28 days prior to chemotherapy.

Results: Long-term tolerance to donor skin grafts was achieved when mice were transplanted with whole BM; T cell-depleted BM or purified LSKs. However, the ability to induce thymic tolerance with this protocol was diminished with age and was not restored with endocrine modulation, which we have previously shown to enhance immune reconstitution. We found instead that donor engraftment was inhibited in aged mice due to a higher proportion of chemotherapy-resistant BM LSKs.

Conclusions: Our model demonstrates that low-dose chemotherapy can be used in conjunction with T cell-depleting antibodies and transient immunosuppression to induce mixed chimerism and allogeneic tolerance in young mice. Preliminary results indicate that robust levels of chimerism are harder to achieve in the aged mouse, due to low levels of donor engraftment. As hematopoietic growth factors have been shown to increase the sensitivity of stem cells to myeloablative treatment, we are currently investigating if pre-treatment with such factors facilitates the induction of hematopoietic chimerism and thus allogeneic tolerance in aged mice.

P1841

Rebound of lymphoid progenitors after allogeneic hematopoietic stem cell transplantation in humans in the absence of acute graft-versus-host disease

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Purpose/Objective: Allogeneic Hematopoietic Stem Cell Transplantation (HSCT) is an effective treatment for malignant and nonmalignant blood diseases. The long term T-cell reconstitution after HSCT is dependent on patient thymic function and affected by acute graft-versus-host disease (aGVHD). Data in mice showed that T-lineage reconstitution after HSCT is limited by progenitor supply to the thymus. In humans, a subset of $CD34^+lin^-CD10^+CD24^-$ lymphoid progenitors have a very low myeloid potential but can generate B, T and NK lymphocytes. They belong to the most immature thymic population, are present in cord blood, bone marrow and, most importantly, in the peripheral blood in adults.

Materials and methods: We analysed by flow cytometry this $CD34^+lin^-CD10^+CD24^-$ Thymic Seeding Progenitor (TSP) population in the peripheral blood of 39 patients, 3 months after HSCT, in comparison to 15 healthy donors.

Results: The rate of TSP in peripheral blood was more heterogeneous in patients than in healthy donors. TSP counts in peripheral blood from patients without aGVHD or patients with a grade I aGVHD were higher than for healthy donors. aGVHD clearly impacted the number of TSP. Patients without aGVHD ($n = 11$) and patients with an aGVHD grade I ($n = 7$) had significantly ($P = 0.005$) more TSP than patients with severe aGVHD [grade II, III and IV ($n = 21$)]. The number of TSP decreased with the worsening of disease. We didn't observe any effect of recipients' age or conditioning regimen on TSP counts. The source of grafted cells (bone marrow or mobilized peripheral blood stem cells) had a low impact on TSP variation. In patients without aGVHD and with aGVHD grade I, the number of TSP was significantly correlated with the number of $CD34^+$ cell contained in the graft. Functionally, T-cell commitment and progenitor frequency was determined using limiting dilution assay on OP9 3T3-L1 cells? Mean T-cell progenitor frequency of TSP sorted from patients without aGVHD was at least as high as for healthy donors (1/13 versus 1/20, respectively).

Conclusions: In conclusion, at 3 months after HSCT, we observe in patients without aGVHD a rebound of circulating TSP, which retain a T cell potential. Understanding the precise role of these cells in the T cell recovery and the mechanisms of action of aGVHD on TSP could give important clues for improving T-cell reconstitution after HSCT in humans.

P1842

Rejection of a kidney transplant for a lymphocyte alogentigen non — human leucocyte antigens: a case report

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Purpose/Objective: Antibodies anti human leucocytes antigens (anti-HLA), anti-endothelium cells and anti-mayor histocompatibility complex class I chain-related (anti-MIC) are the most frequent antibodies in the humoral rejection of transplanted organs. In this study we present a clinical report of a humoral response against a transplanted kidney associated with an antibody with a non-previously described specificity and presents in peripheral blood lymphocytes.

Materials and methods: A peripherical blood sample was extracted from a 50-year-old renal patient. The serumwas separated for using in the cross-match and DNA was obteined from lymphocytes. The HLA alleles was typed by PCR-SSO. The antibodies specificity was determinated by bead array (Luminex thecnology).

Results: A 50-year-old patient with negative screening for anti-HLA antibodies received a kidney from a partially HLA-mismatched related donor (HLA-A24, A-; B18, B61; DR11; DR3 and HLA-A2, A29, B27, B35, DR11 DR12, respectively). Three days after the transplant, the renal function failed. The biopsy showed a capillary periarteritis compatible with a humoral rejection. However, the deposits of C4d were negative. Likewise, the determination by bead array of anti-HLA and anti-MIC antibodies was negative. However, a flow cytometry cross-match, with serum from the fourth day after transplant, showed positive results. The patient was treated with plasma exchange and intravenous human immunoglobulin injection according with the usual protocol. After seven sessions of plasmapheresis, the renal function was recovered and the cross-match became negative.

Conclusions: In summary, we believe that this patient suffered an acute humoral rejection related with complement non-fixing antibodies with non-HLA, non-endothelium cells and non-MIC specificity but present in peripheral blood lymphocytes. These results suggest that this new alloantigen could have more impact in the rejection of transplanted kidneys. Therefore we propose a second cross match and not only the determination of antibodies anti-HLA using bead array before an acute humoral rejection post-transplant.

P1843
Risk factors for relapse in patients with myeloid malignancies

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Purpose/Objective: The past 10 years, there has been an increase in using reduced intensity conditioning regimens (RIC) in allogeneic stem cell transplantation (SCT) for different diseases. Long term effects of these conditioning regimens on outcome factors like relapse is now being investigated in different studies. In this study, we studied risk factors for relapse in patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS).
Materials and methods: Two hundred and sixty-seven patients transplanted between 1999 and 2011 were included. Most patients had AML ($n = 193$) and 74 had MDS. RIC was given to 92 patients and myeloablative conditioning (MAC) to 175 patients. The RIC regimens consisted mainly of a combination of Fludarabine and Busulphan.
Results: Four different risk factors for relapse were found in univariate analysis.

Unrelated transplantation (33% relapse incidence at 5 years) versus sibling transplants (22%), $P = 0.05$.

Acute GVHD, any grade, (23%) versus no aGVHD (40%), $P = 0.02$.

RIC (37%) versus MAC (26%), $P = 0.02$.

A donor sero-positive for 3–4 herpes viruses (CMV, HSV, VZV and EBV) was correlated with decreased incidence of relapse (27%), compared to if the donor was positive for only 0–2 of these viruses (38%), $P = 0.03$.

Higher relapse risk in unrelated donor SCT is partly explained by the use of ATG in these patients. Especially in unrelated transplants with RIC, ATG in combination with long immunosuppression may have hampered the antileukemia effect. In fact, the highest risk of relapse was found in unrelated transplants with RIC (40%). In sibling transplants, no effect of RIC on relapse was found.
Conclusions: In this study, we could find that the use of ATG in unrelated transplants after RIC was correlated with high risk of relapse. Interestingly, the donor virus serology seems to have an effect on relapse. It may be speculated that donor T-cells with previous virus infections may be more immune reactive. It could also be that virus specific T-cells may cross-react with leukemia cells.

P1844
Role of IL17 following T cell depletion in baby rat keratoplasty

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Purpose/Objective: Corneal transplantation (keratoplasty) is the most frequent and successful form of tissue transplantation in adults (<10% rejections). The rejection rate is dramatically increased in infants younger than 3 years of age (~85% rejections). In children, any corneal opacity should be corrected as early as possible to prevent life lasting reduced vision. We established the keratoplasty baby rat model that reproduces the increased rejection rate in the young experimentally. The purpose of this set of experiments was to analyze the impact of T cells following keratoplasty in baby rats.
Materials and methods: Orthotopic keratoplasty was performed between Fisher donor and Lewis recipient rats. Recipients were 3 weeks of age (keratoplasty baby rat model) and were either treated during 1 week with a T cell depleting (clone R73) or an isotypic antibody. Corneal grafts were monitored clinically until rejection occurred but no longer than 35 days postoperatively. In a second set of experiments, animals were examined until the T cell compartment recovered. Lymph nodes and cornea were analyzed by qPCR for mRNA amounts of IL17, IL6, TGFβ, and RORγt.
Results: We could clearly show that T cell depletion abrogated graft rejection. Infiltration of CD3+, CD4+ and CD8+ T cells in the graft of R73-treated recipients was significantly reduced even after recovery of the T cell compartment. Simultaneously, mRNA levels for IL6, TGFβ, IL17 as well as RORγt was increased in draining lymph nodes of R73-treated animals. Surviving grafts of R73-treated animals also showed increased mRNA levels for IL17 and RORγt.
Conclusions: These results lead to the conclusion that IL17-producing cells may contribute to long-term graft survival following T cell depletion in baby rats.

P1845
Strategies for hematopoietic stem cell donor searching: Implications of haplotypic and allelic HLA frequencies

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Purpose/Objective: The availability of matched donors depends on the frequency of the patient's alleles and haplotypes. Therefore, data regarding HLA distribution for each population are needed to evaluate the donor searching approach. Our objective is to establish what HLA alleles and haplotypes are most common in our area. These results can be used to delineate search strategies for potential donors including other related donor no matching sibling.
Materials and methods: This study includes 184 families residing in Cordoba y Jaen (Andalusia, Spain) with a total of 548 haplotypes analyzed. They were HLA-A, B, C and DRB1 typed by high-resolution (High Res SSP Unitray Kit, Invitrogen, Wisconsin, USA and Inno-Lipa HLA, Innogenetics N.V. Ghent, Belgium).
Results: Only 26 HLA-A, 47 HLA-B, 25 HLA-C and 31 HLA-DRB1 alleles were found. These alleles have been previously described in Caucasians as expected from the geographic origin of the studied families. The distribution of predominant alleles studied herein reveals great similarities with those in Spain, Western European and North American populations. In the analysis of haplotypes, a high number were included within the most frequent haplotypes described in Spain. However, the results reveal that the ten most frequent haplotypes in our population were not the same as in other populations. Presence of infrequent HLA associations, showed a negative value for finding a suitable donor, whereas the presence of haplotypes with a frequency ≥ 1% in our sample was a positive factor influencing donor searching.
Conclusions: The data we present here may help to predict the chance of getting a suitable related donor, and it also suggests strategies for improving success in the search

P1846
Study of luminex SA C1q assay in sensibilized renal patients

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Purpose/Objective: Assessment of rejection risk based on preformed or *de novo* donor specific antibody (DSA) is based on the luminex (LMX) IgG median fluorescence intensity (MFI) strength. Widespread

debate exists over the suitable MFI cutoff value of Single Antigens Beads (SAB) for calling an antibody *positive*. The complement-fixing ability of the antibody, irrespective of IgG MFI strength could be a key component of clinical outcome. Recently, it has been developed a C1q-SAB assay that identifies complement-fixing HLA antibodies with high sensitivity and specificity. C1q is the first step in the classical complement cascade. Thus, we aimed to determine the correlation between IgG SAB and C1qSAB assays in patients in renal waiting list.

Materials and methods: These assays use pooled luminescent beads, each uniquely color code distinguishable and coated with a different purified single HLA class I antigen ($n = 97$).

Samples of immunized renal patients and negative and positive controls by LMX were used for validation. These sera were tested by LMX-IgG and LMX-C1q in parallel for HLA class I.

LMX-IgG assay was performed using SAB kits (LABScreen, Onelambda, CA) according to the manufacturer and analyzed on a Luminex platform (LABScan 100). Data were analyzed by Fusion 2.0 (OL, CA). Normalized MFI values from Fusion software were used to assign positive («real« MFI > 1000) and «possible« (MFI = 500−999). These assay detects all IgG binding antibodies irrespective of their complement-fixing ability.

C1qSAB assay. The serum were inactivated by heating 30 min at 56°C. Five millilitres of serum were incubated with 5 ml of LabScreen SAB for 20 min at room temperature (RT) and then incubated with 5 ml of PE-conjugated sheep anti-human C1q for 20 min at RT, washed twice with 80 ml LMX wash buffer and adquired on the LMX. The data were analyzed using «Raw« MFI values. In C1qSAB assay, antibodies were assigned as «posible« when the first increase more than 33% (but at least 400 MFI) over the prior lower MFI bead was observed.

Results: Comparison of antibody detection revealed no correlation based on MFI levels between IgG SAB and C1qSAB assay. IgG positive sera with MFIs as low as 700 were able to fix C1q, whereas other sera with MFIs as high 14500 did not. In our series, only 34% class I IgG SAB antibodies were also C1qSAB+. In several patients, we detected C1qSAB+ against IgG SAB+ that was surely due to IgM antibodies.

Conclusions: C1qSAB assay can detect IgM antibodies that can fix complement. These data suggest that C1q assay could be an important method to evaluate the pre-transplant virtual cross-match and to define the non-permitted specificities (C1q-fixing) in kidney transplantation.

P1847

T Cell subsets responsible for CMV reactivation control in patients after hematopoietic stem cell transplantation

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Purpose/Objective: Depletion of cellular immunity as a consequence of conditioning before allogeneic hematopoietic stem cell transplantation (HSCT) frequently results in CMV reactivation, which may in turn lead to life-threatening infections and require timely antiviral treatment.

Materials and methods: We have investigated the ex vivo response of CMV-specific CD4+ and CD8+ T-cells to CMV antigen (combined CMV total lysate, pp65 and IE-1 peptide mix) in 191 samples from 118 individuals. We included patients with either high or undetectable viral loads, and those who controlled or did not control their CMV reactivations. All patient subsets were compared to healthy donors. Polychromatic flow cytometric measurements of CD154 (CD40L), intracellular cytokines (IFNγ, IL2), and a degranulation marker (CD107a) revealed the functional status of various T-cells simultaneously.

Results: We found that dual IFNγ/IL2 producing CD8+ T-cells were significantly increased in patients controlling their CMV reactivations (average 0.33%, SD = 0.4%) compared to non-controllers (average = 0.02%, SD = 0.07%). In contrast, CD8+ T-cells that produced IFNγ only were the most abundant subtype but they were present in a substantial number of both, controllers (average 4.36%, SD = 4.8%) and non-controllers (average 1.64%, SD = 3.7%). Hierarchical clustering of distinct functional signatures revealed that polyfunctional CD8+ T-cells were acting in concert with other subsets, whereas the isolated production of IFNγ by CD8+ T cells heralds insufficient collaboration with others. On a subset of patients with reactivation of CMV post HSCT, we have evaluated the sensitivity and specificity of functional signature test ($n = 64$ samples) to predict reactivation control. When dual IFNγ/IL2 producing cells above 0.1% cut-off were considered protective, sensitivity of 75% and specificity 93% was achieved, while IFNγ-only production by more 0.3% cells had sensitivity of 88% but specificity of 73% only.

Conclusions: Our study revealed functional signatures that are useful readout of immune monitoring. Furthermore, our data may modify the interpretation of previous studies that assessed only IFNγ.

P1849

Terminally differentiated CD8+ T cells reduce the risk for acute kidney allograft rejection

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Purpose/Objective: End-stage renal disease (ESRD) is associated with lymphopenia and increased T cell differentiation. This phenomenom may be the cause of the ESRD-related impairment of T-cell immunity but clinical evidence in support for this conclusion is scarce. We tested the hypothesis that a more profound ESRD-related T cell dysregulation reduces the risk for acute rejection (AR) in kidney transplants.

Materials and methods: In a prospective study, 185 ESRD patients receiving a kidney allograft were included and followed for 1−2 years. Prior to transplantation, circulating $CD4^+$ and $CD8^+$ T cells were quantified. T cell differentiation was established by determining the percentages of naïve T cells, central-memory T cells, effector-memory T cells and the highly differentiated Tem cells which have regained CD45RA expression (Temra cells). In addition, the frequency of T cells without expression of the co-stimulatory molecule CD28 was measured. Data from age-matched healthy individuals were used for comparison.

Results: In 47 patients, a biopsy-proven AR occurred. Confirming previous results, the ESRD patients had significantly lower T cell counts with a more differentiated phenotype compared to healthy controls. Patients with AR showed the least signs of T cell dysregulation with significantly higher absolute numbers of CD4 T cells, naïve CD4 and CD8 T cells and less terminal differentiation of memory CD4 and CD8 T cells compared to non-rejecting (NR) patients. For instance the percentage of $CD8^+$ Temra cells was significantly lower ($P < 0.05$) in patients with AR when compared to NR patients, i.e 16% versus 25%, respectively. After multivariate proportional hazard logistic regression analysis, only the frequency of terminally differentiated $CD8^+$ Temra cells (per percent 4% decrease of risk, $P = 0.006$; per tertile 34% decrease in risk, $P = 0.002$) and the number of HLA mismatches (per mismatch 33%, $P = 0.005$) predicted the risk for AR.

The CD28 cell surface expression was lost in over 80% of these $CD8^+$ Temra cells, confirming their status of highly differentiated T cells.
Conclusions: Advanced ESRD-related T cell dysregulation yielding an increased frequency of terminally differentiated $CD8^+$ T cells is associated with less AR after kidney transplantation. This confirms the results of previous studies which indicated that these cells may act as suppressor CD8 T cells and expansion of CD28null CD8 T cells is associated with decreased T cell immunity.

P1852

The magnitude of ischaemia and reperfusion injury of the transplanted liver correlates with distinct adaptive immune responses from the hepatic resident lymphocytes

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Purpose/Objective: During organ retrieval for transplantation, the liver is subjected to ischaemia followed by reintroduction of oxygen supply (reperfusion). This ischaemia/reperfusion (I/R) phase induces a sequence of cellular and molecular modifications which can lead to graft dysfunction, thereby increasing recipient's morbidity. Studies in animal models of liver I/R injury suggest that parenchymal cells release damage-associated molecular patterns (DAMP) which trigger a variety of receptors including Toll-like Receptors (TLR) expressed on antigen presenting cells in the liver. This activation induces the release of pro-inflammatory molecules which initiate a cascade of immune responses, including T-cell differentiation programs. However, it is currently unclear whether the resulting activated immune effectors have a damaging or protective effect on the graft tissue.
Materials and methods: In order to test the impact of the magnitude of I/R injury on the adaptive immunity driven by the graft, we compared phenotypically and functionally liver-resident lymphocytes (LRL) from organs subjected to various degrees of I/R injury. We isolated LRL from either living donors (LD), heart beating donors (donation after brain death or DBD) and non-heart beating donors (donation after cardiac death or DCD). DBD and DCD livers are subjected to more severe I/R injury compared to LD grafts.
Results: We found that in the group of DBD, LRL are enriched in CD8 T cells which exhibit an activated phenotype (CD8+CD69+) suggesting that the inflammatory environment of DBD donors promotes activation of CD8 T subsets. Assessment of cytokine production revealed a significant increase in IFN-g production by CD8 T cells in DBD grafts. Together these data suggest that donor-derived activated CD8 hepatic resident cells may account for the significant increase in the graft morbidity observed in the DBD group of recipients. In contrast to DBD livers, LRL from DCD livers preferentially produce IL-17. The potential source (CD8 or gamma/delta T cells) and the functional significance of this IL-17 secretion are under investigation.
Conclusions: Our data suggest that the magnitude of ischaemic injury leads to distinct adaptive immune responses in the graft which may account for the different rejection and liver dysfunction rates observed in cohorts of liver transplant recipients.

Poster Session: Vaccine Development

P1853
'Omic'-based comparison of different formulation of vaccine DC

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Purpose/Objective: Dendritic cells (DC) based vaccination for cancer immunotherapy has not yet fulfilled the expectations, providing only low clinical response rates. In an attempt to optimise DC production, various protocols for differentiation and maturation of monocytes have been proposed. The aim of this study was a comparison of different DC formulations using transcriptome analysis.
Materials and methods: Monocytes derived from the peripheral blood of healthy donors were differentiated for 2 days in serum free medium in the presence of GM-CSF and IL4. Resulting DC were then stimulated for 18h with the gold standard cytokine cocktail (namely TNFα, IL1β, IL6, and PGE_2) or with alternative cocktails, where TLR ligands are combined with IFNγ alone (the MPLA cocktail: MPLA and IFNγ) or in combination with other proinflammatory cytokines (the pIC cocktail: polyIC, IFNγ, TNFα, IL1β and IFNα) alone (the āMPLA cocktailÒ: MPLA and IFNγ) or in combination with other proinflammatory cytokines (the pIC cocktail: polyIC, IFNγ, TNFα, IL1β and IFNα).
Results: Transcriptomic evaluation via cDNA array revealed almost 500 genes differently expressed among immature and the three differently matured DC. Unsupervised clustering suggested that the gene expression pattern of the gold standard DC represents an intermediate between immature and alternatively matured DC. The canonical pathways mostly upregulated in the alternative DC were involved in interaction with innate and adaptive effector cells and comprised for example many chemokine genes. Quantitative real time PCR and ELISA assays were used to validate the array data in the respective immature and mature DC subsets. In addition, the functional relevance of the differentially expressed chemokine genes in attracting immune cell subpopulations was determined.
Conclusions: The 'omi'-based identification of differentially expressed genes in vaccine DC formulations and their functional relevance will lead to the optimization of cancer immunotherapeutic approaches.

P1854
A fusion protein based pneumococcal vaccine

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Purpose/Objective: The Gram-positive bacterium *Streptococcus pneumoniae* is a major human pathogen, causing otitis media, pneumonia, bacteremia and meningitis worldwide. Currently available vaccines are based on the capsular polysaccharide. Polysaccharide vaccines are poorly immunogenic in those most at risk of disease. More recently developed polysaccharide/protein (PS) conjugated vaccines showed increased efficacy in the at risk group, yet are too expensive for developing countries where the burden of disease is greatest. Serotype replacement and capsular switching post vaccine introduction have shown clear limitations to the long-term potential of these vaccines. There is therefore a need to develop cheap serotype independent vaccines against pneumococcal disease.
Materials and methods: Pneumolysin (PLY) acts as a powerful mucosal adjuvant to induce both systemic and mucosal immunity to proteins genetically fused to PLY after delivery intranasally or subcutaneously. In this work, *S. pneumoniae* virulence factors, PsaA, PspA, PspC and PhtD have been genetically fused to PLY and Δ6PLY, a PLY toxoid that lacks haemolytic activity but retains its immunogenic and adjuvant activity. The immune response to these fusion proteins was measured by ELISA. The ability of these fusion proteins to stimulate protective immunity against infection with *S. pneumoniae* was investigated in a murine colonisation model.
Results: Here we show that proteins genetically fused to pneumolysin (PLY) become capable of generating an antibody response that is not seen when they are administered as a mix. Serum IgG responding to PsaA, PspA, PspC and PhtD were at a level comparable to those produced by parental administration. We also report the protective efficacy of these responses in a murine colonisation model.
Conclusions: Pneumococcal antigens genetically fused to Δ6PLY are immunogenic when administered to mucosal surfaces and generate immunity that is protective in a colonisation model.

P1855
A novel adjuvant system based on insoluble H7 flagellin containing microparticles induces enhanced humoral immune responses in Quil A-adjuvanted vaccines

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Purpose/Objective: The saponin Quil A is a potent adjuvant capable of promoting both humoral and cell-mediated immune responses and has been successfully used in ruminant vaccinations targeting parasite hidden antigens (Hags) where protection is antibody mediated. Protection in these vaccines is critically dependent on the levels of vaccine induced Hag-specific antibodies, as Hags are effectively hidden from the host immune response and no natural boosting of the vaccine induced immune response occurs. In order to develop an enhanced Quil A-based Hag vaccine cable of inducing higher titres of antibody, we evaluated the effect of the toll-like receptor 5 (TLR5) ligand flagellin, which is known to drive Th2-polarized responses to co-administered antigen, on Quil A vaccine induced immune responses to the model antigen, ovalbumin.
Materials and methods: Four groups of sheep ($n = 6$) were immunized with either Quil A plus H7 flagellin (purified from *Escherichia coli* O157:H7) and ovalbumin incorporated into the same insoluble microparticles (H7-OVA-MP), Quil A plus H7 flagellin and ovalbumin incorporated into separate microparticles (H7-MP and OVA-MP, respectively), Quil A plus ovalbumin containing microparticles (OVA-MP) or H7-OVA-MP without Quil A. Prior to immunization, bioactivity of H7 flagellin within the microparticles was confirmed using a TLR5 reporter cell line.
Results: Significantly higher titres of anti-OVA IgG were induced following immunization with H7-OVA-MP plus Quil A or H7-MP + OVA-MP plus Quil A compared to immunization with OVA-MP plus Quil A ($P < 0.01$ and 0.05, respectively), with the highest titres observed in the H7-OVA-MP plus Quil A group. Antibody responses were critically dependent on Quil A as immunization with H7-OVA-MP without Quil A failed to induce an anti-OVA antibody response. OVA-specific lymphocyte proliferation and interferon-γ release by peripheral blood lymphocytes were highest in the OVA-MP immunized group compared to all other groups.
Conclusions: These results suggest that H7 flagellin microparticles skew the immune response generated by Quil A adjuvanted vaccines from a Th1 to a Th2-type response, and by inducing increased titres of antigen-specific antibodies may be improve the efficacy and duration of protection of existing Hag-based vaccines.

P1856

A novel replication impaired virus vaccine strategy is effective against congenital cytomegalovirus infection

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Purpose/Objective: Congenital HCMV infection can lead to serious symptomatic disease including mental retardation and hearing loss in newborn. The greatest risk of congenital HCMV is to mothers who acquire a primary infection. Hence, the impact of a vaccine is potentially substantial. Any proposed intervention therapy for HCMV should be evaluated in a pre-clinical animal model. HCMV is extremely species-specific, making direct study of infection in animal models untenable. The guinea pig is unique as the only small animal model for congenital CMV infection using the species specific guinea pig CMV (GPCMV).
Materials and methods: We investigated a disabled infectious single cycle (DISC) vaccine strategy, where the virus lacks the ability to express an essential gene, except when grown on a complementing cell line. The minor capsid gene (UL85 homolog, GP85) was selected as it is essential for virus capsid assembly but in itself not an importnat immune target. A series of GPCMV mutants were generated via mutagenesis of an infectious BAC clone of GPCMV. In the DISC strain, the GP85 late promoter/upstream intergenic sequence was removed and a tet-off promoter and an upstream SV40 poly A cassette were substituted to place GP85 expression under strict control of a tet-off trans-activating system. Transfection of mutants onto tet-off fibroblast cells resulted in virus from the mutant BAC containing the tet-off promoter (vDISCGP85).
Results: The DISC vaccine immune response was investigated. Animals ($n = 6$) induced an anti-GPCMV antibody response (ELISA titer >1:1260; neut. titer approx. 1:160) and a cell mediated response to the pp65 and IE1 homologs, verified by a novel guinea pig INFgamma ELISPOT assay. Next, female guinea pigs ($n = 15$) were vaccinated (vDISCGP85, 10^3 pfu). All animals seroconverted and were given a booster dose and mated. At late second trimester of pregnancy animals were challenged with a pathogenic strain of GPCMV (1×10^5 pfu). A control group, non-vaccinated pregnant animals ($n = 15$) were similarly challenged. Animals were allowed to go to term and viral load in target organs of pups was analyzed.
Conclusions: Based on live pup numbers in the vaccinated and non-vaccinated groups (94.4% versus 63.6%) the vaccine strategy was successful ($P = 0.002$). Pups from the vaccinated group had zero or reduced viral load in target organs. However, the vaccine failed to prevent brain infection in all pups (11% positive in vaccinated group versus 50% in control) but the outcome was statistically significant and indicates that this is a promising new approach.

P1857

A novel virus-like particle-based anticancer vaccine using the mimotope strategy

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Purpose/Objective: Virus-like particles (VLPs) resemble infectious viruses in structure, are highly immunogenic and were shown to induce vigorous innate and adaptive immune response to antigens exposed on their surface. Due to their immunogenic properties, VLPs are promising candidates for vaccine strategies in serving as versatile scaffold for the delivery of a wide range of vaccine antigens * especially of epitopes that are normally difficult to evoke an immune response. In contrast to viral epitopes, antigenic determinants of cancer antigens can often be solely presented as peptide mimotopes. VLPs are well suited for the display of epitopes, however they have never been used as carrier for mimotopes. This research project deals with the utilisation of influenza VLPs as antigen-carrier platform for the presentation of a cancer-associated antigen mimotope. Immunisation studies in mice should elucidate whether the elicited antibodies are able to recognise their original epitope.
Materials and methods: Four different insertion sites in the antigenic sites A and B located in the globular head domain (HA1) on the influenza hemagglutinin (HA, H3 subtype) were tested for their capability in tolerating insertion of the 14 aa sequence of the peptide mimic. Influenza VLPs were generated using the Baculovirus Expression Vector (BEV) system and analysed by flow cytometry and Western Blot.
Results: We were able to demonstrate that insertion of the mimotope did not greatly interfere with the overall structure of some chimeric hemagglutinins, however the presentation of the mimotope needs to be optimised. In order to perform fine-tuning of the microenvironment of the peptide within the HA-frame, adjacent residues are being randomised in a library approach.
Conclusions: By introducing unique restriction sites downstream the polyhedrin promoter into the baculovirus genome, we created a platform that enables us to directly clone our HA-mimotope amplicon library into the baculovirus genome. This will allow us for convenient high-throughput screening of our clones for optimal binding characteristics of the displayed chimeric protein to the mimotope-specific mAb using FACS. Moreover, the establishment of an optimised eukaryotic expression library system should enable us to optimise the display of any epitope on a VLP carrier and thereby enhance its antigenicity.

P1858

A plant-derived oral vaccine against HBV in a tablet form

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Purpose/Objective: Hepatitis B is still one of the most common human infectious diseases, despite the fact that effective anti-HBV subunit vaccines have been available for 30 years. Oral vaccines, including plant-based formulas, have been being considered as commonly available alternatives or supplements for standard injection vaccines, due to assumed low-cost production and simplified vaccination. The aim of research was to study an appropriate host producer, composition and administration protocol to make an efficient anti-HBV plant-derived oral vaccine.
Materials and methods: HBV antigens were expressed in transgenic lettuce, as confirmed by ELISA, Western blot and electron microscopy observation. Plant tissues bearing antigens were lyophilised, powdered and delivered orally to BALB/c mice as suspension in PBS or converted into tablets administered to humans. Immune responses were analysed by assay of anti-HBs antibodies and FACS analyses of T cells.
Results: Individual native HBV antigens were produced in lettuce at levels 5–200 μg/g FW. Vegetative propagation of superior producers enabled scaling-up multiplication of antigen-bearing material.

Efficiency of freeze-drying and long-term storage of plant tissues ranged vastly (10 * 90%) depending on a particular antigen. Powdered lyophilised tissue facilitated controlled oral administration and enabled conversion into tablets for human vaccination.

Relatively low antigen dosage and extended timing between immunisations correlated with higher responses. However, solely oral delivery of lyophilised tissue containing S-HBsAg induced systemic humoral response in mice at the minimal protection level (≥10 mIU/ml of anti-HBs antibodies) with higher sIgA response in intestinal mucosa and immediate stimulation of Treg lymphocytes. Yet, significant reaction was triggered by combination of injection priming and oral boosting (hundreds mIU/ml). Tablets used as a booster vaccine for humans previously immunised by a classical three-dose injection pattern, increased anti-HBs level from 0 up to 100 mIU/ml or two * threefold when pre-immune anti-HBs were detectable.

Conclusions: The prototype technology for a potent plant-derived anti-HBV oral vaccine has been developed. The study provides some basic insight into immunisation using a tablet formula which would serve as a booster component of a vaccine.

P1860
Adjuvanted influenza vaccines in young and aged BALB/c mice

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Purpose/Objective: Although influenza vaccines are generally safe, they are far from ideal. They have reduced efficacy in the groups most at risk: the very young and the elderly. To address these concerns, the vaccine industry is increasingly turning its attention to the use of adjuvants to enhance the immune responses generated by these vaccines. During the 2009 pandemic H1N1 influenza outbreak, an adjuvanted influenza vaccine was selected for administration to most Canadians; it was formulated with the oil-in-water adjuvant AS03 and contained 25% of the usual antigen dose of vaccine. This study investigated the immune responses generated following immunization with adjuvanted influenza vaccines in aged and young mice.

Materials and methods: We tested two ages of BALB/c mice (2 months, 1.4 years), two doses of Influenza A/Uruguay H3N2 split vaccine (0.75, 3 μg), and two adjuvants (AS03, alhydrogel). Mice were given two intramuscular immunizations 3 weeks apart. Serum was collect before and 3 weeks after each immunization. Three weeks following the final immunization, mice were sacrificed and the immune status was investigated in splenocytes.

Results: The use of an adjuvant increased serum hemagglutination inhibition (HAI) titers compared to vaccination with antigen alone. With both adjuvants, we found no significant difference between using the low or high antigen doses, and at all immunization conditions tested, aged mice produced significantly lower HAI titers than young mice. Furthermore, in aged mice, AS03 was found to be the stronger adjuvant for the induction of influenza-specific cellular immune responses. With AS03, aged mice were able to produce equivalent levels of influenza-specific splenocyte proliferation and cytokine secretion (IL-4, IL-5) as young mice.

Conclusions: Following immunization, aged mice produce lower antibody responses in comparison to young mice; however, the use of the oil-in-water adjuvant AS03 can induce some cellular immune responses in aged mice to levels that are equivalent to young mice. We are investigating additional differences in the immune responses of young and aged mice with a goal of designing more effective vaccines, especially for the elderly.

P1861
Analysis of the vaccine induced immune response against the abomasal parasite *Ostertagia ostertagi* in cattle suggests a pivotal role for NK cells

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Purpose/Objective: Control of gastrointestinal nematodes by vaccination would offer a valuable alternative to the use of anthelmintic drugs. However, the development of such vaccines is largely hampered by a lack of knowledge on the protective immune responses needed against these parasites. In this study, the immune responses to three different experimental vaccines against the abomasal nematode *Ostertagia ostertagi* in cattle were analysed and compared: the protective native ASP (nASP) + QuilA vaccine, and two non-protective versions of this vaccine, one combining nASP with $Al(OH)_3$ and one combining recombinant ASP (rASP) with QuilA.

Materials and methods: Three groups of four animals were immunized with the vaccines three times intramuscularly with 3-week interval. After the final immunization, all animals were challenged with 1000 infective L3 larvae/day until necropsy 5 weeks later. Mononuclear cells (MC) isolated from peripheral blood (PB) were phenotyped weekly during the experiment and their *in vitro* proliferation to the vaccine antigens analysed. At necropsy, MCs from the abomasal mucosa and lymph nodes (LN) were collected and analysed as described above and mucosal antibody levels induced by the vaccinations were measured.

Results: No significant changes in frequencies of ab T cells, gd T cells, natural killer (NK) cells and B cells in PB were observed following vaccination. Both nASP/QuilA and rASP/QuilA vaccination resulted in detectable *in vitro* proliferation of PBMC to vaccine antigens, with markedly the highest proliferation in the nASP/QuilA vaccinated animals. The proliferating cells were identified as NK cells. At necropsy, no significant changes were observed in frequencies of ab T cells, gd T cells, NK cells and B cells in the abomasal mucosa and LN. Although MC isolated from the abomasal LN of all animals responded to nASP, likely caused by the challenge infection, proliferation was highest in the nASP/QuilA vaccinated group, again with NK cells being the strongest responders. Analysis of the mucosal antibody response indicated a significant increase in ASP-specific IgG1 levels in the nASP/QuilA vaccinated animals.

Conclusions: The outcome of this study suggests a previously unidentified role for NK cells and IgG1 antibodies in vaccine induced immunity against gastrointestinal parasites.

P1862
Antigen delivery by ß-sheet aggregated protein potentiates efficient immune response

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Purpose/Objective: The accumulation of β-sheet aggregates, such as amyloids or amyloid-like proteins, is a hallmark of protein misfolding diseases as this structure element is absent in natively folded proteins. Although the role of the immune system in the progression or control of these diseases has been widely studied, the connection between β-aggregation and its immunological effects remains unclear. Here we demonstrate that β-sheet aggregates act as important adjuvant of cellular immune responses.

Materials and methods: We used the well-established model antigen ovalbumin (OVA) and its transgenic mice for our study. Employing a

standardized thermic misfolding technology, we induced b-sheet aggregates in OVA (cbOVA). Uptake was accessed by confocal microscopy and flow cytometry; while processing and presentation was measured by cytokine release and cellular proliferation. Blocking antibodies to scavenger receptors (SR) and SR-A knockout mice were used to determine the receptor mediating the uptake.
Results: cbOVA was generated by misfolding native OVA thermically. DCs that were pulsed with cbOVA showed improved antigen presentation via both MHC class I and class II pathways, enabling OVA specific $CD4^+$ and $CD8^+$ T cell priming, proliferation and Th1-like cytokine production. cbOVA is taken up more efficiently in DC, but it does not maturate DC nor does it induce inflammasome activation. Pre-incubation of cbOVA with specific b-sheet aggregation structure ligand FnF4-5 would inhibit its uptake and presentation. Moreover, coupling the $CD8^+$ T cell epitope to b-sheet forming peptides induced robust T cell response. Finally, we found uptake and presentation of cbOVA is mainly mediated by SR-A.
Conclusions: We demonstrated that β-sheet aggregation significantly improves immunogenic properties of misfolded proteins and peptides by enhanced uptake into DCs via a SR-mediated fashion. Furthermore, we identified SR-A as one of the major receptors that mediates the stimulatory effect of b-sheet aggregation structure. Given the notion that controllable b-sheet aggregation is inducible in most proteins, we propose that our fundamental study may be highly relevant for future vaccine design; paving ways to a widely applicable, simple and reproducible technique.

P1863
Antigen size impacts on duration of antigen presentation and dictates Tfh development

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Purpose/Objective: Revealing the fundamental processes controlling the initiation, maintenance and phenotype of an adaptive immune response will make a significant contribution to rational vaccine design. Previous approaches have overcome the inherently low immunogenicity of subunit vaccines through combination with particulate delivery systems e.g. biodegradable polymers and liposomes. While each delivery system has its own merits, it is unclear how changing the physical characteristics of a vaccine antigen could impact on the magnitude and duration of antigen persistence and presentation *in vivo*, and the impact this has on the subsequent adaptive immune response. By modulating delivery of antigen to the LN simply by changing particle size, we aimed to establish the functional outcome for specific T and B cell responses and the potential mechanisms underlying this.
Materials and methods: Proteins covalently linked to inert nanospheres of varying size were used as particulate antigen. The EαGFP protein combined with the monoclonal antibody YAe were used to determine kinetics of peptide/MHCII presentation. An established adoptive transfer model using TcR transgenic T cells and BcR transgenic B cells was used to study functional antigen specific responses.
Results: Challenge with a 200 nm particulate antigen promoted sustained peptide/MHCII presentation by DCs. 200 nm antigen also promoted differentiation of T follicular helper cells (Tfh), supported germinal centre responses and enhanced antibody production. Smaller particulate (40 nm) and soluble antigen were able to drive T cell proliferation but without sufficient differentiation of Tfh cells to support germinal centre or antibody responses.
Conclusions: With the increased focus on using particulate delivery systems to target LN resident cells in vaccination strategies, our data reveal a rationale for antigen delivery strategies to promote high affinity class switched antibody responses.

P1865
Apolipoprotein B-100 peptide conjugate confers tolerogenic properties to dendritic cells

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Purpose/Objective: We have previously shown that immunization with an apolipoprotein B peptide vaccine (aBp210), with peptide 210 coupled to cationized BSA (cBSA), decreases the development of atherosclerosis with up to 60%. Further studies have demonstrated the involvement of T regulatory cells (Tregs) as a part of the protective mechanism. Moreover, our studies also point towards an independent role of cBSA in reducing atherosclerosis. Therefore, we investigated the effect of these individual vaccine components on dendritic cells (DC), $CD4^+$ T effector cells and Tregs in an *in vitro* setting to further elucidate the protective mechanism behind the aBp210 vaccine.
Materials and methods: $CD11c^+$ DCs, T effector cells ($CD4^+CD25^-$) and Tregs ($CD4^+CD25^+$) are isolated from mouse C57Bl6 spleens. T effector cells are pre-activated using plate bound anti-CD3 and DCs plus Tregs are co-cultured together with antigen (p210-cBSA, cBSA or BSA) over night. The pre-activated T effector cells are then cultured with DC/Tregs for 72 h and proliferation is measured after 16 h of [3H]Thymidine incorporation.
Results: Results from our *in vitro* assay demonstrate that both cBSA and p210-cBSA when pre-incubated with DCs and Tregs overnight are able to inhibit the proliferation of anti-CD3 activated T effector cells in a dose dependent manner. The suppressive effect of cBSA was dependent on the cationization as BSA in itself was without effect. Adding the antigen directly to polyclonally activated T effector cells did not suppress proliferation. Moreover, the inhibition of proliferation was associated with a dose dependent decrease in IL-12 production and addition of IL-12 to the culture system could completely remove the inhibitory effect of p210-cBSA.
Conclusions: Our findings suggest that p210-cBSA induce tolerogenic dendritic cells by inhibiting their IL-12 production in a dose dependent manner. This might be one of the protective mechanisms behind aBp210s role in inhibiting atherosclerosis and vascular inflammation. cBSA is a common carrier used in vaccine formulations such as aBp210. Here, we are also able to show a direct immune-suppressive effect of cBSA, reducing the proliferation of T effector cells. These observations suggest that cBSA is an attractive carrier in vaccines aiming to induce tolerance.

P1867
Association of Th17/Treg in NALT with pneumococcal carriage in children and adults

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Purpose/Objective: Background: CD4+CD25+ Regulatory T (Treg) cells play an important role in the regulation of the immune response to infection, whereas Th17 have been suggested to be crucial in bacterial clearance from the host. In this study, we have studied the association between the ratio of Th17/Treg cells and nasopharyngeal carriage of pneumococcus in children and adults. We have also studied

the induction of those cells by pneumococcus in human nasal-associated lymphoid tissue (NALT).
Materials and methods: Methods: Mononuclear cells (MNC) from adenotonsillar tissues were isolated from children and adults undergoing adenotonsilectomy. Treg and Th17 cells were enumerated in adenotonsillar MNC by intercellular staining of Foxp3 and IL 17 with or without stimulation by concentrated pneumococcal culture supernatant (CCS) derived from a type II pneumococcus D39. IL-17 production in adenotonsillar MNC was measured by ELISA.
Results: There was a significant difference in the proportion of Th17 in adenotonsillar MNC between adults and children, which was higher in the former in whom pneumococcal carriage was low. The same trend was also shown in the ratio of Th17/Foxp3+ Treg between adults and children. There was a significant increase in both Treg and Th17 in adenotonsillar MNC after stimulation by the pneumococcal CCS. Significant increase in IL17 production in adenotonsillar MNC after CCS stimulation was shown by ELISA.
Conclusions: It is suggested that the development and balance of Th17 cells and Foxp3+ Treg in the local mucosal immune tissues play an important role in modulating the specific immunity against pneumococcal carriage in humans.

P1868

Cellular and humoral immunity elicitated by influenza vaccines in pediatric hematopoietic-stem cell transplantation

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Purpose/Objective: Immunity induced by influenza vaccines following hematopoietic stem-cell transplantation (HSCT) is poorly known.
Materials and methods: In this case serie study, proliferative T-cell and humoral responses to influenza vaccines evaluated vaccine immunogenicity in 14 pediatric recipients (mean age: 6 years). Cases were vaccinated against the 2009 H1N1 pandemic strain (H1N1pdm2009), either alone ($n = 9$) or in association with the seasonal trivalent vaccine ($n = 5$). Median lag time from HSCT to vaccination was 5.7 months. The nature of HSC was HLA-identical related bone marrow graft in 10/14 recipients. Fourteen age-matched non-vaccinated recipients were included as controls. In addition, cytokine (IL2 and IFN-gamma) responses to influenza were evaluated by an intracellular accumulation method in part of the recipients.
Results: Vaccinees opposed to non-vaccinated recipients evidenced higher proliferative responses to H1N1 ($P = 0.0001$; median stimulation index: 42 versus 1; median lag time from HSCT to investigation: 335 days in both groups) and higher titers of antibodies specific for the 2009 H1N1 pandemic strain ($P < 0.02$; geometric mean: 160 versus <10; median lag time from HSCT to investigation: 107 days). Specifically, 11/14 (79%) vaccinees evidenced proliferative responses to H1N1, of whom 5/7 evaluable had protective (>1/40) antibody titers. Five out of seven recipients vaccinated against H1N1 but not against H3N2, evidenced proliferative responses to both H1N1 and H3N2 strains (median stimulation-index: 96 and 126 respectively). Finally, IL2 responses predominated over IFN-gamma responses ($P < 0.02$) to influenza viruses in responders.
Conclusions: Influenza vaccination elicitated substantial cell-mediated immunity and to a lesser extent protective humoral immunity at early times post-HSCT in this pediatric series. Monovalent H1N1pdm2009 vaccine induced cross-reactive T-cell responses to seasonal H1N1 and H3N2 strains. Protective (IL2) rather than effector (IFN-gamma) cytokinic profiles were elicitated.

P1869

Cellular immune response to influenza A (H1N1) pandemic vaccine in kidney transplant (KTR) or HSCT recipients, HIV infected and SLE immunocompromised populations

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Purpose/Objective: Influenza vaccines are recommended for immunosuppressed patients. Cellular response to vaccines is essential for a long term protection, but has not been characterized in these patients. We evaluated the cell responses to the inactivated influenza A (H1N1)v 2009 vaccine in different immunocompromised patients.
Materials and methods: The study included 125 patients receiving: A one or two doses of influenza H1N1 pandemic vaccine with adjuvant in 36 treated HIV-infected patients [18 with maraviroc, a CCR5 antagonist (MVC), and 18 without] and 30 HSCT recipients B two doses of the non-adjuvanted vaccine in 29 KTR and 30 SLE patients. H1N1v-specific CD4 and CD8 T cells producing IFNγ, IL2, TNFα, MIP1β or expressing CD40L were evaluated at D0, D21, D42 and M6/M9 of vaccination using intracellular cytokine staining with overlapping H1N1v 2009 hemagglutinin peptides.
Results: In group A, frequencies of H1N1v-specific CD4, but not CD8, T cells significantly increased after one dose at D21 and M6 in non-MVC patients ($P = 0.023$, $P = 0.021$) but not in MVC patients, and only after two doses in HSCT patients ($P = 0.04$). Only frequencies of H1N1v-specific CD8 T cells at D21 differed between the non-MVC patients and HSCT recipients ($P = 0.03$). Similar proportions of responders, defined as twofold increase in frequencies of H1N1v-specific T cells from D0 to D21, D42 or M6/M9, were identified in 38%, 50% and 50% for CD4 and 61%, 55% and 45% for CD8 T cells in MVC patients, non-MVC patients and HSCT recipients respectively. In group B, a significant increase in frequencies of H1N1v-specific CD4 T cells were detected from D0 to D21 in SLE patients ($P = 0.02$) but not in KTR. Proportions of responders were twofold higher in SLE (70%) than in KTR (34%) for CD4 T cells and 70% versus 51% for CD8 T cells in SLE and KTR patients respectively. Among responders, a significant increase in frequencies of H1N1v-specific CD4 and CD8 T cells was observed until M9 in KTR ($P = 0.01$ and 0.007) but not for SLE patients.
Conclusions: The pandemic influenza A-H1N1v vaccine, whether adjuvanted or not, induced only modest T cell responses in these different immunocompromised populations. Patients characteristics seem to influence both short and long term protection and the benefits of the adjuvant or the 2nd dose, indicating new strategies are required to improve the immunogenicity of vaccination in these immunocompromised patients.

P1870

Co-stimulatory potency of Interleukin 2 applied as 'natural adjuvant' bound to virus-like nanoparticles is critically influenced by its membrane-anchor characteristics

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Purpose/Objective: Decoration of VNP with functionally active, immunomodulatory molecules enhances particle immunogenicity and

promotes selective immune recognition of the antigens they bear. In this study we investigated the influence of membrane characteristics of artificially membrane-bound cytokines applied as 'natural adjuvants' to improve the immunogenicity of VNP co-expressing viral antigens.
Materials and methods: One (1Ig), two (2Ig) or four (4Ig) immunoglobulin (Ig)-like domains of CD16b were inserted between the model cytokine Interleukin 2 (IL 2) and the minimal GPI-anchor acceptor sequence of CD16b (GPI). We compared targeting of IL 2 fused to different membrane anchors to lipid rafts and VLP and tested the influence of the membrane anchor on the biological activity and co-stimulatory potency in antigen-specific and non-specific assays.
Results: We identified a membrane anchor * '2IgGPI' * conferring an up to 10-fold increase of targeting of IL 2 onto VNP, when compared to the minimal GPI-anchor acceptor sequence. This effect was particularly prominent in cases where additional molecules were targeted onto VNP and space seemed to be restricted. When co-expressed on VNP with H2-D^b presenting the lymphocytic choriomeningitis virus glycoprotein peptide 33−41 (LCMV-GP_{33-41}), as antigen-specific signal 1, IL 2::2IgGPI was superior to IL 2::GPI to co-stimulate proliferation of primary LCMV-GP-specific P14 TCR transgenic T-cells *in vitro*, particularly when antigen was limited. Correspondingly, the proportion of IFNγ producing and $CD107^+$ $CD8^+$ antigen-specific T cells was significantly increased. Upon *in vivo* challenge of mice with antigen-specific VNP co-expressing IL-2::2Ig-GPI larger proportions of adoptively transferred LCMV-specific T-cells proliferated when compared to IL-2::GPI co-expressing VNP. Corresponding loss-of-function variants of IL-2 induced consistently less pronounced T-cell proliferation.
Conclusions: The requirements for the optimal biological activity of artificially membrane-anchored cytokines decorating VNP used as convenient immunization platform cannot be predicted *a priori* but has to be evaluated *in vitro* and *in vivo* with scrutiny.

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P1871
Comparison of the efficacy of protein and adeno−based influenza vaccines targeting M2e

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Purpose/Objective: There is an urgent need for a more practical influenza vaccine that can be used not only during seasonal epidemics but also in the event of occasional pandemics. Whereas recent focus has been placed on targeting conserved sites on the major glycoprotein hemagglutinin (HA), due to the variability of this protein, it faces the dilemma of only inducing group specific protection. The highly conserved ectodomain of the M2 protein (M2e) has been presented as a promising alternative target since it is conserved amongst all human strains. Our previous results showed that a protein mimicking the tetrameric conformation of M2e (named M2e-NSP4) was superior to an M2e peptide in immunogenicity at low protein concentrations and at providing local early protection. In this complementary study we made a comparison between the M2e peptide, the tetrameric M2e vaccine, and two adeno viral based vaccines encoding three M2e's linked to the invariant chain and the whole M2 sequence respectively.
Materials and methods: Balb/c mice were immunized with either M2e peptide or the M2e-NSP4 protein. In a separate experiment, balb/c mice were primed twice with DNA-gold encoding three M2e's linked to the invariant chain (M2e × 3Ii) and the whole M2 (M2) sequence respectively, and then boosted with adenoviral vaccines encoding the same antigen. The mice were challenged with a lethal dose of influenza A PR8 2 weeks the adenoviral immunization. Serum samples from both the peptide/protein and the adenoviral vaccine experiment were analyzed in a M2e peptide based ELISA assay and in a HeLa-M2 cell based FACS assay.
Results: Interestingly, DNA-gold priming twice followed by adenoviral vaccine boost using either antigen design, induced an increased number of antibodies recognising the native M2 protein as presented on HeLa-M2 cells compared to either M2e peptide and M2e-NSP4 vaccine. However, in a PR8 challenge model comparing only the adenoviral vaccines, the M2 group was inferior to the M2e × 3Ii group.
Conclusions: The results presented here, suggest that a protective correlate of M2e based vaccination cannot simply be deduced from M2 binding, but are potentially influenced by other factors such as isotype of IgG and/or T cell responses.

P1872
CrossTope: a curate repository of three-dimensional structures of MHC: epitope complexes

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Purpose/Objective: The CrossTope is the first repository of three-dimensional structures of peptide: MHC complexes, including curated data on immunogenicity, similarity relationships and cross-reactivity.
Materials and methods: The complexes hosted by this databank were obtained in protein databases and by large-scale *in silico* construction of MHC: peptide structures, using a new approach (D1-EM-D2 − *Docking1-EnergyMinimization-Docking2*) developed by our group. In both modeled structures and those acquired in databases, we included only non-redundant structures containing immunogenic epitopes with experimental evidence supporting this feature (in the case of two deposited structures in proteins database from the same MHC: peptide complex, we chose the structure presenting better resolution).
Results: At this moment the database contains 182 *non-redundant* MHC: epitope complexes (169 models and 13 crystals) from two human (HLA-B*27:05 and HLA-A*02:01) and two murine alleles (H2-Kb and H2-Db). A web server (http://www.crosstope.com.br) provides interface for database query. The user can download the (1) structures files in .pdb extension (2) topology and charge distribution files (-5 and -10 kiloteslas), in .jpg format, from the TCR-interacting surface of MHC: epitope complexes. The files can also be viewed online. The retrieved structures and topological maps can be used to cluster similar epitopes in cross-reactivity approaches (i.e. using HCA − Hierarchical Cluster Analysis), to analyze viral escape mutations in a structural level or even to improve the immunogenicity of tumor antigens.
Conclusions: In this way, CrossTope database opens a way for the exploration of an additional level of complexity on immunogenic epitopes, the comparison at the molecular level, hitherto confined to analysis of scarce pMHC complexes.

P1873
Cytokine-mediated modulation of coronavirus vector-induced antitumor immunity

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Purpose/Objective: Efficient vaccination against infectious agents and tumors depends on specific antigen targeting to dendritic cells (DCs).

The use of viral vectors represents a superior strategy to elicit both innate immune activation and optimal induction of T cell immunity. We recently described a novel biosafe coronavirus-based vaccine approach that facilitates delivery of viral or tumor antigens to DCs *in vivo* (Cervantes-Barragan *et al.*, 2010, MBIO e00171-10). Since the microenvironment during T cell priming is of great importance for the outcome immune response (i.e. memory or effectors activities), we designed vectors containing either 'myeloid' (Flt3L) or 'lymphoid' cytokines (IL-2 or IL-15) together with a viral model antigen.
Materials and methods: Vectors were produced from cloned cDNA using purified vaccinia virus DNA and cytokine production after vector administration was measured by ELISA. Lewis lung carcinoma and B16 melanoma cells encoding for the viral model antigen were used to assess both prophylactic and therapeutic vaccination regimen.
Results: Single immunization with only 10^5 cytokine-encoding coronavirus-based particles was sufficient to elicit vigorous expansion of antitumor $CD8^+$ T cells, and to achieve prophylactic and therapeutic tumor immunity. Importantly, the Flt3L-encoding vector elicited superior immune responses response showing protective antitumor immunity at doses as low as 100 particles.
Conclusions: Taken together, we have developed a novel coronavirus-based vaccine approach that facilitates antigen delivery to DCs and allows for immune-modulation through expression of different cytokines.

P1874
Development of a therapeutic vaccine against chronic Hepatitis C virus infection

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Purpose/Objective: Approximately 170 million people worldwide are chronically infected with Hepatitis C virus (HCV). There is no effective vaccine available. Treatment with a combination of pegylated interferon-a and ribavirin, which is associated with a high toxicity, is effective in <50% of these chronically infected patients. HCV resolution in acute patients is correlated with vigorous and broad spectrum of both CD8 and CD4 T cells responses demonstrating that the cellular immune system in principle has the potency to develop an effective anti-HCV response. The aim of our study is to increase and broaden HCV-specific immune responses to control viral replication and eliminate the virus.
Materials and methods: We developed a novel immunization strategy based on a viral vector derived from the alphavirus, Semliki Forest Virus (SFV). We generated different SFV replicon particles encoding various part of non-structural proteins (nsPs) of HCV. The expression of nsPs in infected BHK21 cells was measured by ^{35}S-methionine labeling. While the immune-stimulating potency of these rSFV replicon particles was studies *in vivo* by prime and boost intramuscular immunizations in C57BL/6 mice. IFNg production, degranulation and CTL activity of HCV-specific T cells were analyzed *ex vivo*.
Results: Here we show that these SFV replicon particles *in vitro* produce significant amount of nsPs. Furthermore, mice immunized with SFV replicon particles encoding either all nsPs or NS3/4A induces comparable amount and activity of HCV-specific cytotoxic T cell against an HCV NS3 peptide.
Conclusions: In this study we demonstrate that SFV replicon particles can encode foreign nucleotides up to 6 kb (all nsPs) without affecting the immunogenicity of the proteins expressed. This may enable our goal to broaden the HCV-specific response. By augmenting both breadth and strength of HCV-specific cellular immune response, HCV-infected cells could be eliminated, lowering the viral burden. In summary, we show that an SFV vector-based vaccine is a potential candidate for the induction of wide and robust HCV-specific cellular response. Further analysis on the specificity of T cells against other nsPs epitopes will be performed. Next we will explore strategies to augment antiviral efficacy that do not affect healthy liver tissue.

P1875
Development of an improved recombinant MVA vaccine vector able to induce strong innate and adaptive immune responses

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Purpose/Objective: Vaccines against cancer and pathogens such as HIV and Plasmodium falciparum need to induce high numbers of multi-functional antigen-specific CD8 T cells. While replicating viral vectors efficiently induce CTL responses, safety concerns restrict their usage. A combination of good CD8 T cell inducing capacities and a favorable safety profile is given with MVA-BN®, a replication deficient derivative of CVA.
Materials and methods: In order to further enhance innate and adaptive immune responses after MVA immunization, we generated a recombinant vector expressing a TNF superfamily member and the model antigen Ovalbumin.
Results: This new vector amplified DC activation and cytokine synthesis *in vitro* and *in vivo*. Antigen-specific primary and memory CD8 T cell responses were drastically enhanced. Importantly, the generated CTLs also showed improved functionality as demonstrated by intracellular cytokine staining and *in vivo* killing activity. The superior CTL responses were able to protect B cell deficient mice against a fatal virus infection.
Conclusions: Taken together, we developed a new recombinant vaccine vector, able to induce strong CTL responses with enhanced functionality.

P1876
Evaluation of adenovirus-based single antigen-administration schedules for immunization against retrovirus infection in mice

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Purpose/Objective: Adenovirus (Ad)-based vectors are very potent tools for the induction of strong CTL responses, and we have developed a so-called expression-display vector that induces strong $CD4^+$ T cell and antibody responses against an antigen that is encoded and displayed on the Ad capsid. As repeated administrations of Ad vectors can quickly give rise to anti-vector immunity, we aimed at establishing a vaccination protocol based on single administrations of the respective vectors. Experiments were performed in the Friend retrovirus model in mice.
Materials and methods: Highly FV susceptible mice were immunized with an adenoviral vector encoding a fusion protein of murine thioredoxin and an FV CTL epitope, or with an adenoviral gp70 expression-display vector that encodes and displays a fusion protein of FV Env gp70 and the Ad capsid protein pIX. Each vector was administered once, either following one another or as a single immunization in the presence of pre-existing anti-Ad immunity. Immune responses and protection from FV challenge were analyzed.
Results: The order in which vectors were administered proved crucial for the vaccination outcome. The single application of a CTL inducing vector is sufficient for induction of a robust CTL response, but is significantly hampered if the other vector was applied first. Antibody

responses induced by the gp70 expression-display vector were not hampered by anti-vector immunity, but were actually enhanced, probably due to help from anti-Ad $CD4^+$ T cells. The combination of a first immunization with a CTL inducing vaccine, followed by immunization with an expression-display vector, proved to be the most effective and conferred a high degree of protection against high-dose FV challenge.

Conclusions: We present a potent vector combination that is highly effective for immunization against retrovirus infection.

P1877

Evaluation of baculovirus production in three insect cell lines for standardized gene delivery in humans and animals

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Purpose/Objective: The Baculovirus and insect cell expression system has been efficiently used for the production of heterologous proteins and recently gained importance as gene-delivery platform for gene therapy and vaccination in humans and animals.

Aside the systems' benefits, there still remains a bottleneck in determining the baculovirus titer in a reproducible way. Usually, baculoviral products almost exclusively rely on the *Spodoptera frugiperda* (Sf9) insect cell line for titer determination. It has been shown that virus generated in Sf9 cells, which was subsequently titered on Sf9, High Five and the BTI-*Tnao38* insect cell line, yielded different results (Hashimoto *et al.*, 2010). We propose that there exist differences in respect to the cell line used.

Materials and methods: The above mentioned 3 insect cell lines BTI-*Tnao38*, High Five and Sf9 were infected with various baculovirus constructs. Viral titer was determined on all the three cell lines by plaque assay and endpoint dilution assay. Real-time PCR was performed to determine the overall number of viral particles.

Results: Comparison of the three insect cell lines showed cell line dependent effects. Differences were found in respect to infection with different Multiplicities of Infection (MOIs) and subsequent harvesting at various time points. The baculovirus envelope protein gp64 was found to be less present in virus generated in BTI-*Tnao38* and High Five cells compared to Sf9.

Conclusions: Cell line dependent variations are suggested to function as basis to gain further insights on determination of the accurate dose of baculovirus-derived vaccine and gene-therapy products and may support the manufacturing of a baculovirus reference material for inter-laboratory comparison.

P1879

Genetically engineered chimeric molecules targeting influenza A viral antigen

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Purpose/Objective: The use of live and attenuated strains of microbes and viruses for vaccination comprises the risk of evoking B and (or) T cell epitopes possessing undesirable characteristics. This necessitates the application of epitope-specific vaccines. DNA vaccination using naked DNA encoding viral antigens has the potential to induce both humoral and cellular immune responses as live attenuated viral vaccines do. We hypothesized that sequences encoding an epitope of virus hemagglutinin (IP) attached to sequences encoding a scFv antibody fragment against co-stimulatory cell surface receptors (complement receptors 1/2 on mouse B cells or FcγRI on human monocytes) would result in the *in vivo* expression of a chimeric viral peptide with increased immunogenicity.

Materials and methods: Gene and protein engineering; Protein expression; DNA immunization; ELISA; Cytotoxic assay.

Results: The DNA constructs were engineered by linking of sequences encoding the scFv antibody fragment against the appropriate receptor and IP peptide representing a conserved epitope of influenza A virus hemagglutinin. Each DNA construct ends with either human oncogene Fos or Jun. We have inserted the DNA constructs into protein expression vector system and used them as naked DNA vaccine. Serum antibodies against the viral peptide from DNA injected animals were measured with ELISA. The cell-mediated immune response induced in the animals was evaluated using a cytotoxic assay.

An immunization with a DNA plasmid containing the described construct induced a strong anti-influenza cytotoxic response lasting for more than 6 months. After prime-boosting with protein chimeric molecule we obtained anti-influenza cytotoxic and antibody response.

Conclusions: Immunization of mice with pure DNA, encoding the antigen of interest attached to a scFv antibody fragment to positive receptors, followed by prime-boosting has been successfully used to induce protective immunity against a model pathogen. An analogous type of engineered molecule could be used for the treatment of other experimental viral or bacterial infections and different peptide epitopes could be used.

P1880

Glycosylation matters: role of viral hemagglutinin *N*-glycosylation in immunogenicity

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Purpose/Objective: One of the major players in influenza virus virulence is the glycoprotein hemagglutinin (HA). Most influenza vaccines target HA to elicit protective immunity. Innovative influenza vaccine production in mammalian cell lines as an alternative to conventional virus propagation in chicken eggs gets more and more into focus. Besides virus strain and subtype, the host cell line used for influenza virus production determines HA *N*-glycosylation. Due to an increasing demand for cell-line based influenza vaccines it is of special interest in which way differential glycosylation of viral proteins affects their immunogenic properties.

Materials and methods: In this study, we investigated the impact of the differential *N*-glycosylation patterns of two influenza A PR/8/34 (H1N1) variants on immunogenicity. Splenocytes from TCR-HA transgenic mice (all T cells express a TCR$\alpha\beta$ specific for the $HA_{110*120}$ peptide presented by $I\text{-}E^d$ MHC class II molecules) were stimulated with MDCK cell-derived (M-variant) or Vero cell-derived (V-variant) influenza virus preparations. Cytokine production and the expression of T cell activation markers were analyzed to determine the impact of HA *N*-glycosylation on immunogenicity. Next-generation pyrosequencing validated the congruence of the potential HA *N*-glycosylation sites and the presence of the $HA_{110-120}$ peptide. Additionally, influenza virus preparations were natively deglycosylated by a mixture of endo- and exoglycosidases to confirm that observed effects were glycosylation-dependent. Furthermore, mice were immunized with the fully glycosylated as well as the deglycosylated influenza glycovariants.

Results: TCR-HA transgenic splenocytes stimulated with the V-variant up-regulated the T cell activation marker CD69 faster and produced significantly more IL-2 than cells stimulated with the M-variant. Similar results were obtained *in vivo*. Co-cultivation assays with dendritic cells (DCs) and T cells indicated that this difference in immunogenicity was mediated by $CD11c^+$ DCs. Native virus deglycosylation dramatically reduced cytokine production by splenocytes.
Conclusions: In conclusion, we show that influenza HA *N*-glycosylation markedly affects immunogenicity. This finding might have important implications for cell-line based influenza vaccine design.

P1882

Identification and generation of CD8 T-cell subsets required for vaccine protection against Ebolavirus

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Purpose/Objective: The objective of the studies described is to define T-cell responses that are required for vaccine protection of nonhuman primates (NHP) against lethal Ebolvirus challenge. These data are used to guide development of vaccines that generate both rapid and durable T-cell based protective immunity.
Materials and methods: Single-shot vaccines based on replication-defective adenovirus 5 (rAd5) induce potent antigen-specific humoral and cellular responses against Ebola virus and protect NHP challenged 4 weeks post vaccination. Depletion of $CD8^+$ T cells eliminates protection, demonstrating that rapid generation of T cell responses is necessary for virus clearance. We used vaccine vectors derived from several adenoviruses (human and chimpanzee) with unique receptor usage and APC targeting, and that exhibit various degrees of protection against Ebola to elucidate the development, magnitude, and phenotype of $CD8^+$ T cells needed for protection.
Results: We present data demonstrating that the magnitude of vaccine-induced $CD8^+$ T cells does not predict protection. We show, rather, that immune protection requires the generation of a specific subset of effector T-cells defined by their pattern of intracellular cytokine secretion (quality); survival associates specifically with $CD8^+$ T cells that produce a combination of two cytokines, TNF-alpha and IFN-gamma. In contrast, 'polyfunctional' T cells that secrete these cytokines in combination with IL-2 and are thought to represent optimal immunologic memory are rare or absent from vaccines that protect against Ebola challenge.
Conclusions: The need for effector T cells that can mobilize rapidly is consistent with the biology of Ebola virus which is tropic for all tissues and fatal within 10 days of exposure in nonhuman primates, but presents a challenge for development of a vaccine that provides both rapid immunity and durable memory. We show strategies to overcome this problem in vaccine development and define the dynamics that govern generation of long term effector T cell-based protection.

P1883

Identification of immunodominant T cell epitopes from anthrax protective antigen for inclusion in a rationally designed sub-unit based vaccine

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Purpose/Objective: A major determinant of *Bacillus anthracis* pathogenicity is a binary exotoxin composed of Protective Antigen (PA) and 1 of 2 subunits, Lethal Factor (LF) or Edema Factor (EF). The PA protein, which consists of four domains, remains the most extensively characterised of the anthrax toxins, and was an early focus of work to identify an antigen that provided protective immunity to anthrax. However, to date, studies have focused almost exclusively upon serology and the number of PA antigens that have been defined as T cell immunogens is very limited.
Materials and methods: Using a panel of humanised HLA-DRB1*0401 transgenic mice, we have defined HLA-DR restricted CD4+ T cell responses to the immunodominant epitopes of PA presented following immunisation. PA antigen-specific T cell hybridoma panels were also screened for recognition of the immunodominant peptides. This was correlated with the responses of two human cohorts; individuals vaccinated with the Anthrax Vaccine Precipitated (AVP) vaccine and patients recovering from cutaneous anthrax infections.
Results: Although a number of immunodominant CD4+ T cell epitopes were identified, the response was predominantly biased towards an epitope located in the C terminus domain IV of PA, which is essential for PA binding to receptors on the host cell surface. HLA binding studies also revealed that this peptide bound across a number of common HLA-DR alleles with remarkably high affinity.
Conclusions: Based upon this data we therefore selected the immunodominant PA epitopes for inclusion in an epitope-string vaccine construct, which was evaluated in HLA transgenic mice challenged with live *B. anthracis*. The protective effect observed indicates that the identification and inclusion of CD4+ T cell epitopes will be central to the success of future 'next generation' vaccines against bacterial pathogens such as *B. anthracis*.

P1885

Identification of RNA-based vaccine adjuvants through systematic fragmentation of the Influenza A nucleoprotein sequence

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Purpose/Objective: The purpose of the study was to identify viral RNA sequences with high immunostimulatory potency selected based on cytokine induction in human peripheral blood mononuclear cells (PBMCs) and selective TLR induction.
Materials and methods: Various RNA sequences of different length derived from Influenza A virus nucleoprotein (NP) gene were tested fortheir potential to stimulate PBMCs and HEK293 cells transfected with human TLR3, 7, 8 and 9 (TLR-HEK cells). All RNA fragments

were applied in equimolar concentrations. PBMCs were transfected with cationic liposome-complexed RNA and IFNa as well as TNFa secretion was determined by ELISA 16 h post transfection. Transfection of TLR-HEK cells with a plasmid carrying a luciferase reporter gene under the control of an NFkB-inducible promoter allowed simple and sensitive monitoring of TLR signaling induction. TLR-HEK cells were transfected with RNA complexed with liposomes and NFkB induction was measured by standard luciferase assay 16 h post transfection.

Results: Among three fragments of equal length (525 nt) together representing the full-length NP sequence, A2 led to higher amounts ofIFNa and TNFa secretion from PBMCs than the two flanking fragments (A1 and A3). Comparison of A2 with the full-length NP sequence in TLR-HEK cells showed higher stimulatory capacity of A2 as revealed by higher orders of luciferase induction in TLR3 TLR-HEK cells. No increased induction of TLR signaling was observed in TLR7, 8 and 9 TLR-HEK cells. In order to investigate whether the higher stimulatory potential of A2 was due to its diminished length or rather to specific regions within the sequence, three subfragments of equal length (175 nt) were tested in TLR3 TLR-HEK cells. Whereas TLR3 induction by two subfragments, A2-2 and A2-3, remained below that of A2, subfragment A2-1 was able to reach the same level as A2. Most interestingly, upregulation of luciferase gene expression after addition of the other two subfragments to A2-1 was superior to A2 alone.

Conclusions: These results imply that the nucleotide sequence determines the extent of immunostimulatory properties of RNA fragments rather than their length. Further investigations on the nature of such sequences may help to design optimized RNA-based vaccine adjuvants.

P1887

Immunogenicity of DNA vaccines encoding simian immunodeficiency virus antigen targeted to dendritic cells in rhesus macaques

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Purpose/Objective: Targeting antigens encoded by DNA vaccines to dendritic cells (DCs) in the presence of adjuvants enhances their immunogenicity and efficacy in mice.

Materials and methods: To explore the immunogenicity of this approach in non-human primates, we generated a single chain antibody to the antigen uptake receptor DEC-205 expressed on rhesus macaque DCs. DNA vaccines encoding this single chain antibody fused to the SIV capsid protein were delivered to six monkeys each by either intramuscular electroporation or conventional intramuscular injection co-injected or not with poly ICLC, a stabilized poly I:C analogue, as adjuvant.

Results: Antibodies to capsid were induced by the DC-targeting and non-targeting control DNA delivered by electroporation while conventional DNA immunization at a 10-fold higher dose of DNA failed to induce detectable humoral immune responses. Substantial cellular immune responses were also observed after DNA electroporation of both DNAs, but stronger responses were induced by the non-targeting vaccine. Conventional immunization with the DC-targeting DNA at a 10-fold higher dose did not give rise to substantial cellular immune responses, neither when co-injected with poly ICLC.

Conclusions: The study confirms the potent immunogenicity of DNA vaccines delivered by electroporation. Targeting the DNA via a single chain antibody to DEC-205 expressed by DCs, however, does not improve the immunogenicity of the antigens in non-human primates.

P1888

Immunological analysis of 35 new antigens of *M. paratuberculosis* in mice infected with *M. paratuberculosis*, *M. avium*, *M. tuberculosis* and *M. bovis*

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Purpose/Objective: To evaluate the immunogenicity and the specificity of 35 novel *M. paratuberculosis* proteins in mice experimentally infected with *M. paratuberculosis*, *M. avium*, *M. tuberculosis* and *M. bovis*.

Materials and methods: Eighteen of these 35 candidates were identified in standard *M. paratuberculosis* biofilm cultures (surface pellicle) on Sauton medium (Leroy *et al.*, 2007) and 11/35 were identified in latency models based on Sauton cultures submitted to different stress conditions (hypoxia, acidic pH, nutrient starvation or non-toxic NO). Proteomic and immuno-proteomic analyses were performed on culture filtrates and bacterial pellets. Finally, 6/35 were identified using an *in silico* analysis of *M. paratuberculosis* genome.

The 35 genes were cloned in *E. coli* expression plasmid pQE-80L (Qiagen) and IPTG-induced histidine-tagged recombinant proteins were purified by affinity chromatography on immobilized nickel-chelate (Ni-NTA) columns.

Mice were infected intravenously with *M. bovis* AN5 and with three luminescent strains *M. paratuberculosis*, *M. avium* ATCC 15769 and *M. tuberculosis* H37Rv obtained by transformation with pSMT1 plasmid.

At day 1 and 2, 5, 8, 12, 20 and 28 weeks after infection, mice were killed and organs were individually analysed for bacterial burden by luminometry and by/or standard plating. Spleen cell IFN-γ production following *in vitro* stimulation with the 35 proteins and serum antibody levels were measured by ELISA as described before (Roupie *et al.*, 2012).

Results: Among the 35 proteins, 11 induced an IFN-γ production as early as 2 weeks post-infection and this number increased to 26 at 20 weeks post-infection. IFN-γ responses were negative in response to nine proteins.

Among the four species, *M. avium* was the most virulent strain as reflected by very high bacterial numbers in spleen, lungs and liver. *M. tuberculosis*/*M. bovis* bacterial burden was highest in lungs, whereas highest MAP numbers were detected in liver.

Conclusions: The immunogenic antigens identified in this study may be relevant for early, specific diagnosis of bovine paratuberculosis and for MAP vaccine development.

P1890

Immunotherapy of bladder cancer with BCG cell wall skeleton encapsulated sub-200 nm nano particles

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Purpose/Objective: Intravesical administration of live BCG is a gold standard for therapy of carcinoma *in situ* and superficial carcinoma. Live BCG seems to infect to tumor cells and induce immune responses against tumor cells. In other words, it is essential for induction of efficient antitumor immune responses that live BCG is taken up by bladder tumor cells. Although an effective therapy, the serious side-effects associated with using live mycobacteria for treatment, such as fever, granulomatous infection, sepsis and even death, pose a significant concern. Therefore, it is necessary to develop a more active but less toxic immunotherapeutic agent.

BCG cell wall skeleton (BCG-CWS) is an adjuvant component of live BCG and has been expected as bladder cancer vaccine instead of live BCG. However, BCG-CWS has problems in pharmaceutical preparation and low cellular affinity because of poor solubility, strong-negative charge and huge molecular weight. Thus, delivery systems are needed to deliver BCG-CWS into bladder tumor cells. In this study, we incorporated BCG-CWS into octaarginine-modified nanoparticles (R8-NP), our original delivery system, for the pharmaceutical properties and delivery into bladder tumor cells. In addition, we also investigated the antitumor effect of the nanoparticles against bladder carcinoma.

Materials and methods: The BCG-CWS incorporated R8-NP (R8-NP/CWS) was composed of phosphatidylcholine, cholesterol, stearylated octaarginine and BCG-CWS. Cellular uptakes of R8-NP/CWS into mouse bladder carcinoma cells (MBT-2) were evaluated by flow cytometer and confocal laser scanning microscopy. Antitumor effect against MBT-2 in mouse model was performed by inoculating a mixture of R8-NP/CWS and MBT-2 cells. Rat model which naturally developed bladder cancer was prepared by feeding carcinogen. After bladder tumor developed, R8-NP/BCG was intravesically administered.

Results: R8-NP/CWS was <200 nm in diameter and water-soluble preparation. R8-NP/CWS was efficiently taken up by MBT-2. Moreover, R8-NP/CWS significantly inhibited tumor growth in mice bearing MBT-2. Finally, R8-NP/CWS was intravesically administered to the rat model. As the consequence, R8-NP/CWS significantly inhibited the growth of bladder cancer.

Conclusions: In conclusion, the R8-NP/CWS is a promising drug against bladder carcinoma instead of live BCG.

P1891

Impact of a novel conjugation method on the efficiency of CD40mAb-antigen conjugate vaccines *in vivo*

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Purpose/Objective: The interaction of CD40, expressed by antigen-presenting cells, with CD154, expressed by activated T cells, leads to the initiation of humoral and cell-mediated immune responses. Antibodies against CD40 (CD40mAb) act as CD40 stimulants mimicking T cell help. Conjugation of CD40mAb to antigen enhances the immunogenicity of the antigen by up to 1000-fold and improves anti-tumour protection following vaccination.

Conjugates made in our previous studies using sulphydryl-maleimide coupling were poorly-defined and heterogeneous, containing both high and low molecular weight (MW) products. We hypothesize that a purer, low MW conjugate would be a more effective vaccine. To address this we have investigated the use of an alternative conjugation strategy that employs click chemistry and have compared this to our previous methods.

Materials and methods: CD40mAb was chemically crosslinked to the model antigen ovalbumin (OVA). (1) In the standard method, CD40mAb was treated with *N-Succinimidyl* S-*acetylthioacetate* (SATA) introducing a protected sulphydryl bond and the antigen was maleimide-activated using Sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC). (2) In the click chemistry method, CD40mAb was functionalised on lysine residues with an alkyl cyclooctyne and reacted with azide functionalised antigen to form a stable triazole. Click chemistry conjugates were separated via size-exclusion chromatography (HPLC) into several fractions, primarily very high MW conjugate and low MW conjugate. The components of unfractionated conjugate and fractions were analysed by Western blotting and sandwich ELISA using anti-rat IgG to capture and anti-OVA to detect. Flow cytometry on CD40 transfected L929 fibroblast cells was used to assess binding to native CD40.

Results: Western blotting showed both CD40mAb and OVA were present in high and low MW material, indicating conjugate formation. We show that low MW conjugates were >30 fold more efficient when compared to the higher MW conjugates in the sandwich ELISA described above. Murine studies comparing pure high and low MW conjugate for immunogenicity and protection against OVA expressing B16 melanoma tumours will be presented.

Conclusions: Low MW conjugate shows promise as a more potent vaccine construct.

P1892

Induction of HIV-1 neutralizing antibody and cell-mediated immune responses by using multimeric E2 scaffolds co-administered with DNA

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Purpose/Objective: It is widely believed that induction of both broadly neutralizing antibodies (bNAbs) and cell-mediated immunity are crucial for a successful HIV-1 vaccine. In order to elicit both humoral and cellular immune responses against HIV-1 we generated multimeric scaffolds, based on the acyltransferase component (E2) of the pyruvate dehydrogenase complex of *Geobacillus stearothermophilus*, displaying antigenic determinants from the HIV Envelope (Env) glycoproteins gp120 and gp41.

Materials and methods: E2 self-assembles into 60-mer virus-like particles (VLPs) with 60 copies of an antigen on each scaffold. The HIV-SF162 V3 and MPER regions were expressed as *N*-terminal fusion to the E2 core. To increase solubility, Env-E2 VLPs were refolded from inclusion bodies with equimolar amounts of E2 in stepdown dialysis. The assembled 60-mer particles were purified by size exclusion chromatography and were >90% pure, as assessed by SDS-PAGE and Western blot. Identity was confirmed by 447-52D and 2F5 binding to Env-E2 monomers. Env-E2 multimers were tested alone and in combination with HIV-SF162 Env (gp160) plasmid DNA in rabbits and in mice. NAbs were measured using TZM-bl assay; T-cell

responses were determined by dextramer staining, intracellular cytokine staining and thymidine proliferation assays.
Results: Immunization of rabbits with V3-E2 VLPs co-administered with DNA elicited strong V3-specific NAb response after two immunizations in 6 weeks. Co-immunization of V3-E2 VLPs plus DNA elicited V3-dextramer specific CD8+ T cells producing IFNg in mice. Rabbits immunized twice or more with DNA plus MPER-E2 VLPs rapidly generated strong NAbs, and a subset was specific for 2F5 epitope. Immunization of mice with MPER-E2 plus DNA induced CD4+ T cells that specifically proliferated and produced TNF-alpha.
Conclusions: Combination VLP plus DNA approach elicited strong anti HIV-1 Env NAb responses and cell-mediated immune responses. Our results further substantiate this novel strategy of co-immunization with scaffolds and DNA for vaccination against HIV-1, and indicate that this approach could represent an adaptable and efficient platform by which to direct immune responses toward conserved regions of Env.

P1893
Induction of the enduring T cell memory against *M. tuberculosis* on vaccination with peptide of 16 kDa antigen linked to Pam2Cys

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Purpose/Objective: In TB-endemic populations, one of the main reasons for the failure of BCG vaccine is the obstacle caused by environmental mycobacteria in its processing and presentation to T cells. Usually, peptides do not require extensive antigen processing since they directly bind to major histocompatibilty complex molecules and can be presented to T cells. Therefore, peptide vaccines can surmount the problems associated with BCG failure. Therefore vaccine that does not require extensive processing can be quite successful in TB-endemic regions. Hence we have developed a construct by linking promiscuous peptide of *Mycobacterium tuberculosis* to Pam2Cys, a Toll-Like-Receptor-2 agonist. This vaccine has self-adjuvanting property, elicits long-lasting T cell memory and provides better protection than BCG.
Materials and methods: Protection studies in mice and Guinea pigs. Animals (7–10/group) were immunized as described above and rested for 75 days. Animals were then exposed to *M. tuberculosis* H37Rv through aerosol route at 100 CFU (mice) or 30 CFU (Guinea pigs) and sacrificed 30 days later. Mycobacterial burden in lungs was estimated by CFU plating. For histopathological analysis, formalin fixed tissues were processed and stained with hematoxylin and eosin.

Staining for surface markers on DCs and T cells. Cells were harvested and incubated with Fc block and stained with anti-mouse fluorochrome/biotin labeled mAbs for 30 min/4°C. Usual steps of washings were followed after the each incubation. Cells were fixed in 1% paraformaldehyde. The data were acquired using FACS-Aria II.

T cell Proliferation. It was monitored by 3H-thymidine incorporation and CFSE-dye-dilution assay.

Cytokines secretion: These were estimated by ELISA and intracellular-cytokines-staining.
Results: The vaccine construct can be targeted to dendritic cells to secrete cytokines, induces and enhances the expression of costimulatory molecules and increases their antigen presentation ability and help to T cells. Vaccine can bind directly to major histocompatibilty molecules and imparts enduring Th1 memory response and elicits better protection than BCG in the mouse and Guinea pig model of tuberculosis. It surmounts major histocompatibilty barriers and evokes immune response irrespective of genetic polymorphism of healthy individuals, tuberculosis patients and their contacts.
Conclusions: Lipidated peptide has self-adjuvanting property and activates both innate and adaptive arm of immunity. It generates enduring T cell memory response and induces better protection than BCG. Hence this lipopeptide can be a potent future vaccine candidate against tuberculosis.

P1894
Influence of vaccination strategies on IgG Fc glycosylation

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Purpose/Objective: Induction of IgG antibodies after vaccination plays an important role in the protection against pathogens. Antibodies can neutralize the interaction of the pathogen with a specific receptor on target cells but are also involved in the elimination of the pathogen. The pathogenic potential of IgG antibodies is thereby highly dependent on the IgG subclass and on IgG Fc glycosylation. IgG Fc defucosylation highly increases the effector function of IgG antibodies to recruit killer cells in tumor therapy and IgG Fc de-galactosylation and de-sialylation increases the inflammatory potential of IgG antibodies as described e.g. for arthritis patients. We sought to examine the IgG Fc glycosylation after different vaccination protocols.
Materials and methods: We administered chicken ovalbumin (OVA) under different vaccination protocols to mice and analyzed OVA-reactive IgG Fc glycosylation. The pro- or anti-inflammatory function of differentially glycosylated anti-OVA IgGs was further investigated *in vivo*.
Results: Stimulation with antigens under inflammatory conditions induces de-sialylated IgGs. In contrast, tolerance induced immunosuppressive sialylated IgGs that were sufficient to block antigen-specific T and B cell responses, DC maturation and inflammation.
Conclusions: Our data show that a pro-inflammatory IgG Fc glycosylation pattern is highly dependent on the vaccination protocol.

P1895
Innate and adaptive immune responses to plant-based influenza H5 virus-like-particle vaccines by murine splenocytes

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Purpose/Objective: A novel H5N1 influenza vaccine candidate has been developed, based upon H5-bearing virus-like-particles (VLP) produced in tobacco plants (*Nicotiana benthamiana*). These VLP have demonstrated strong immunogenicity in both pre-clinical and clinical models, but their mechanism of action has still not been fully characterized. The immunologic microenvironment generated by innate responses to vaccination has been shown to greatly influence subsequent adaptive immune responses. The goal of this study was to characterize both the early innate and adaptive H5-specific cytokine responses in mouse splenocytes elicited by these plant-based VLP.
Materials and methods: To study innate responses to H5 VLP, splenocytes from H5-naïve 6–8 week old female Balb/c mice were isolated and exposed to 10 mg/ml H5 VLP. For adaptive immune responses, 6–8 week old female Balb/c mice were given two doses of

H5 VLP (2.5 μg/ml based on H5 content), 3 weeks apart. At study termination, harvested splenocytes were stimulated with recombinant H5 protein (5 μg/ml). In both models, culture supernatants were sampled at various times post-stimulation and cytokine/chemokine levels were measured by multiplex ELISA.
Results: *In vitro* stimulation of antigen-naïve mouse splenocytes by H5 VLP induced a predominantly pro-inflammatory response, with significant increases in five cytokines/chemokines (IL-6, MCP-1, TNF-α, MIP-1α, and CCL5) versus unstimulated controls. For adaptive immune responses, influenza-specific cytokine analysis focused on IFN-γ, TNF-α, and IL-2 levels, as these cytokines have been associated with better disease outcome in different models. Splenocytes from the vaccinated mice secreted high levels of IFN-γ and IL-2 in response to H5 protein restimulation but no TNF-α was detected. Secretion kinetics varied between the different cytokine/chemokines.
Conclusions: This work suggests that plant-based influenza H5 VLP vaccines elicit an early pro-inflammatory cytokine/chemokine response in splenocytes isolated from antigen-naïve mice. These innate effectors may serve to prime for a more potently anti-viral adaptive response upon re-exposure to influenza antigens, characterized by high levels of IFN-γ and IL-2.

P1896
Interaction between OAdV7, a novel ovine adenoviral vector and human immune cells

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Purpose/Objective: Identification of adenoviral isolates of non-human origin has fostered development of vectors with vaccine potential that overcome the problem of pre-existing immunity in the human population that may limit clinical applications. Ovine adenoviral isolate, OAdV287 (OAdV7), the prototype of the genus Atadenovirus, has been characterized as a gene delivery vector. Previously we reported the use of recombinant OAdV7 as a vaccine for inducing an antitumor immune response in a mouse model and showed immunization with OAdV7-OVA prevented and retarded tumor growth in prophylactic and therapeutic tumor trials, respectively. Here we present data describing the impact of OAdV7 vector on human immune cells.
Materials and methods: Monocyte-derived Dendritic cells (mono-DCs) were treated with OAdV7 or hAdV5 for 48 h and transduction was assessed by the percentage of eGFP+ cells. For DC maturation, DCs were stained with antibodies for surface markers CD80, CD86, CD83 and HLA-DR and analyzed by FACS. Supernatants from DCs incubated with viral vectors were analyzed for cytokine concentration using CBA.
Results: Mono-DCs incubated with OAdV7 were successfully transduced using a high multiplicity of infection (MOI). Treatment of Mono-DCs with OAdV7 produced up-regulation of co-stimulatory markers CD80, CD86 and activation marker CD83, HLA-DR. Induction of DC maturation was greater with OAdV7 treatment than with hAdV5 although with hAdV5 transduction appeared greater at the same MOI. Cytokine analysis showed that OAdV7 induced secretion of TNFα, IL-6, IL-12/23 p40 and IL-12 from treated mono-DCs.
Conclusions: This is the first report on the ability of OAdV7 to transduce and activate human antigen presenting cells. The data justify further studies to evaluate the utility of this vector for tumour immunotherapy.

P1897
Lipooligosaccharides study of *Neisseria meningitidis*: the immunotype L2 expression in relation to serogroups in Brazil

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Purpose/Objective: The profile of antigen expression among meningococci is important for epidemiology surveillance and vaccine development. *Neisseria meningitidis* may be classified according to the lipooligosaccharide immunotypes. The types of LOS expressed by a bacterium may influence the behaviour and the fate of the strain. Our study represents the opportunity to focus on a particular aspect of the imunotypes of *N. meningitidis* strains of L2 immunotype epitope present in serogroups A, C, W, Y and B of Brazil .
Materials and methods: To this end two new mouse monoclonal antibodies (MAbs) have been produced against *Neisseria meningitidis* lipopolysaccharide (LPS). The MAbs were reactive against outer membrane antigens and present bactericidal activity. They were tested against different meningococcal strains 118 strains of serogroup A, 66 strains of serogroup C (1972−1974) 293 strains of serogroup B (1992) and 120 strains (2011−2012) of B, C, W135 and Y by Dot-ELISA .
Results: The MAbs were reactive against different meningococcal strains 118 strains of serogroup A, 66 strains of serogroup C (1972−1974) and 293 strains of serogroup B (1992) meningococci by Dot-ELISA. The prototypes and subtypes of B:4:P1.15, B:4:P1.9, B:4:P1.7, B:4:P1.14, B:4:P1.16, B:4:NT and B:NT:NT were detected in *N. meningitis* B serogroups. The strains C:2a:P1.2 and A:4.21:P1.9 were dominant in the C and A serogroups respectively. FACS analysis showed that the MAbs immunotypes recognized LPS immunotypes on the surface of *N. meningitidis*. The immunotype L_{379} was strongly expressed in 90% of *N. meningitidis* B compared with 67% in serogroup C and 18% in serogroup A, whereas L_1 and L_8 were weakly to moderatly expressed by 7% and 15% in serogroup B and 3% serogroup A and was not expressed in serogroup C. Interestingly the prevalence of imunotype L2 is related to serogroups A and B analyzed. Our results demostrated that the expression of LPS (immunotypes) in the *N. meningitidis* Brazilian strains studied is heterogeneous and related to serotypes present in diferent immunotypes.
Conclusions: Our study represents also the opportunity to focus on a particular aspect of the L2 immunotype strains of serogroup A, C B, W135 and Y of Brazil . Consequently, it can be hypothesized that these strains have a compensatory system explaining their ability to cause invasive disease. The explanation may be multi-factorial, as MenB possesses a whole battery of possibilities to escape the host's immune system as capsular switching event.

P1899
Microneedles mediated delivery of tumour antigens to Langerhans cells *in vivo* is an effective vaccination strategy for tumor immunotherapy

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Purpose/Objective: Dendritic cells (DCs) are professional antigen presenting cells capable of generating strong T cell dependent primary immune responses, including cytotoxic CD8 T cell responses, necessary for effective anti-tumour immunity. Recently, several studies have highlighted the complexity of the cutaneous DC network, indicating that due to the high number of DCs present in the epidermis and dermis, the skin might represent the ideal site for vaccine delivery.
Materials and methods: In this study, we used polymeric dissolvable microneedle arrays (MNs) laden with a model antigen (ovalbumin)

encapsulated nanoparticles (NPs) to target specific skin DCs subsets and explore CTL immune responses.
Results: We confirmed that following *in situ* uptake, skin derived DCs were able to deliver NPs to cutaneous draining lymph nodes where they consequently induced potent activation of antigen specific IFN-γ secreting- CD4+ and CD8+ T cells. Moreover, we validated functional relevance of the induced immune responses in murine models of Sendai and melanoma. We further investigated the capacity of different skin DCs subsets to cross-present antigen after OVA-NPs immunization through MNs. Although Langerin+ dermal DCs induced minor OT-I T cell proliferation, Langerhans cells (LCs) constituted the main subset capable of cross-presentation. We confirmed a critical role for LCs in the immunization process when depletion of LCs at the time of MNs immunization dramatically reduced the proliferation of antigen specific CD8 T cells. Furthermore, we investigated the contribution of LCs for the generation of antitumorimmune responses after MNs immunization and found that depletion of LCs significantly reduced the tumour protective effect.
Conclusions: Therefore, we show that direct targeting of antigen to the specific skin DCs subsets, in particular LCs, through MNs vaccination can be successfully utilized to induce robust antitumourimmunity.

P1900

Modulation of experimental Sjögren's syndrome and diabetes via mucosal vaccination with fusion proteins derived from cholera-toxin and heat shock protein 60 kDa

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Purpose/Objective: Cholera-toxin based CTA1-DD and CTA1R7K-DD direct immune responses due to their differential enzymatic activity. The aim of the study was to investigate their potential in modulating spontaneous diabetes and Sjögren's syndrome (SS) in non-obese diabetic (NOD) mice via heat shock protein 60 kDa peptide aa437−460 (aa437-460) fusion proteins.
Materials and methods: NOD mice were immunized intra-nasally with fusion proteins at 7-weeks of age. Untreated NOD and BALB/c mice were used as controls. Alterations in antigen presenting cell (APC) and effector cell populations were analyzed by flow cytometry 1-week post immunization. Diabetes incidence was determined from 12 weeks of age onwards and SS disease phenotype was assessed at 7-, 11-, 21 and 24-weeks of age.
Results: In NOD, neither significant change of cell population sizes nor of co-regulatory receptor and ligand expression levels were observed in response to vaccination. Mice administered with CTA1R7K-aa437-60-DD exhibited accelerated and increased diabetes incidence, whereas vaccination with CTA1-aa437-460-DD caused the opposite effect. Regarding SS, salivary and lacrimal gland secretion capacity at 11-, 21- and 24-weeks of age remained unaffected.
Conclusions: In contrast to previous results, the cholera toxin-based fusion proteins did not induce expansion of specific APC populations. Irrespective of this lack in immune adaptation, the diabetes incidence curves suggest that modulation of autoimmunity is peptide dependent and reflects the inherent properties of aa437-460. In addition, the results also reflected a difference in fusion protein function. The similarities in exocrine gland function between treated groups compared to untreated NOD and BALB/c controls probably reflect that overt disease has yet to manifest itself. However, target organs have been collected and prepared for histological assessment of inflammation and potentially microarray analysis; these will be used for determination of adjuvant effect.

P1901

Mouse model and protein candidates- finding an effective vaccine against *Chlamydia trachomatis*

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Purpose/Objective: *Chlamydia trachomatis* caused genital infections are one of the most frequent sexually transmitted diseases worldwide with more than 90 million new cases occurring each year. Typical disease sequelae include cervicitis, endometritis, salpingitis, but also abortion and infertility. Recurrent infections increase the likelihood of these complications. Although chlamydial genital tract infections can be cured with antibiotics, a definitive control of *C. trachomatis*-caused diseases (STDs) is only possible through the development of a safe and efficacious vaccine.
Materials and methods: Therefore we try to establish the necessary mouse model using the *C. trachomatis* strain UW-3/Cx, serotype D. Furthermore, to produce the effective vaccine, we define, clone, express and purificate the required proteins and finally test them *in vivo*. A number of immunogenic antigens have been tested for their ability to induce protection; we decided to use the major outer membrane protein (MOMP) at one hand and the polymorphic outer membrane proteins (Pmps) on the other hand. Firstly, we tested the antigens alone and secondly in combination with a variety of immunostimulatory adjuvants. For investigating *C. trachomatis* infections we had a closer look on different mouse strains (Bl6/Balb/c/C3H), which we infected with different amounts of chlamydial infection forming units (IFUs).
Results: After 7 days of infection the mice, which were infected with 2×10^6 or more (IFUs) showed a plain redness and thickness of the uterus horns and the tubes. These macroscopically visible clinical signs of disease will be used to evaluate the success of the vaccine. Furthermore, the clearly enlarged lymph nodes will serve as source of B-, T- and antigen-presenting cells to investigate the immunologic reaction provoked by the infection and induced by the vaccine.
Conclusions: In summary, the aim of this project is not just to reduce the bacterial load after infection with *C. trachomatis*, but to prevent disease and to induce long- term immunity.

P1902

Novel RSV genetic vaccine based on chimpanzee Adenovirus and MVA vector is safe and highly immunogenic in rodents and primates and fully protects cotton rats from RSV challenge

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Purpose/Objective: Respiratory Syncytial Virus (RSV) infection is the main cause of hospitalization of infants and young children in industrialized countries. RSV affects also the elderly where treatment of infection is only supportive. Thus, RSV vaccine will have a tremendous impact and might be used in all infants and other risk groups.

We generated several replication incompetent chimpanzee-derived Adenovirus (ChAd) for the delivery of vaccine antigens. ChAd vectors are insensitive to pre-existing immunity and have been validated in Phase I and IIa clinical trials where they showed good safety, immunogenicity and efficacy. ChAd can be used in heterologous prime/boost regimens with Modified Vaccinia Ankara (MVA) vectors encoding for the same antigens leading to high immune responses.

Materials and methods: To induce a full spectrum of RSV-specific immune responses including neutralizing antibody and CD8+ T cells, we generated ChAd and MVA vaccine vectors encoding for the F, N and M2-1 RSV antigens. The vaccine was tested in pre-clinical animal models including mice, non-human primates (NHP) and cotton rats by different regimens and routes (intramuscular and intranasal), including mucosal delivery by intranasal spray in NHP. T cells responses were assessed by *ex-vivo* IFNg-ELISpot and Intracellular Staining assays, while humoral responses were tested by ELISA and Neutralization assay.
Results: The vaccine induced high levels of local and systemic T cell immunity in mice and NHP (up to 7200 and 13 500 SFC/10^6 splenocytes or lung cells and 8000 SFC/10^6 PBMC, respectively) with no signs of toxicity. High titers of neutralizing antibodies were induced in both species with levels exceeding by 100 fold those measured in protected human adults and infants.

In cotton rat model, the vaccine completely prevented replication of the virus in the lung and in the nasal cavity. Lung histopathology analysis showed that the vaccine was safe and didn't induce enhanced respiratory disease. GLP toxicology study in mice showed no vaccine related safety concern and GMP grade RSV genetic vectors have been prepared and are ready to be tested in clinical trials.
Conclusions: These data demonstrate that our RSV genetic vaccine is safe, induces a full range of immune responses in mice and NHP and fully protects cotton rats from RSV challenge.

P1903
Novel vaccine strategy for ocular surface infections using conjunctiva-associated lymphoid tissue as a route of immunization: local and systemic responses to model antigen

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Purpose/Objective: Ocular surface infections include several blinding disorders that affect the cornea and conjunctiva at the front surface of the eye. Since antimicrobial therapy has shown its limitation to protect from reinfection and vision threating sequels (i.e. Trachoma) also partially protective vaccines against ocular surface pathogens would impact ocular morbidity. As mucosal immune responses (e.g. nasal, oral, gastrointestinal) are most efficiently induced by the administration of vaccines onto mucosal surfaces, we aimed to assess the efficacy of the ocular mucosa with the conjunctiva-associated lymphoid tissue (CALT) as a mucosal route of immunization. The aim of the present study was to elucidate local and systemic immune responses after CALT immunization using a model antigen.
Materials and methods: BALB/c and C57BL/6 mice were immunized via conjunctiva with tetanus toxoid (TTd) as a model antigen (100 μg/mice, 5 μl were applied onto conjunctiva). Two percentage glycerol and merthiolat-inactivated *B. pertussis* were used as adjuvants. Subcutaneously immunized mice were used as a 'gold standard' (100 μg/mice, 100 μl). Three immunizations were performed at 2 weeks interval and the evaluation of local and systemic immune response was done 2 and 8 weeks after the last immunization. Protection assay against tetanus toxin (2 LD_{50}) has been done a month after the last immunization.
Results: We have found TTd-specific IgG and IgA in tears and sera of both mice strains, as well as, IgG positive TTd-specific cells. It was shown that the amount of TTd-specific antibodies in mouse sera strongly correlated with the presence of TTd-specific B cells in draining lymph nodes. Eight weeks after the last immunization, the levels of anti-TTd IgG and IgA in the sera of BALB/c mice were higher than in C57BL/6 although C57BL/6 exhibited more prominent IgA concentration in mouse tears. *B. pertussis* displayed adjuvantic properties when added to TTd as it was revealed that TTd-specific IgG and IgA immune responses in this mice group were the highest in both mice strains. Mice were challenged with a lethal dose of tetanus toxin by the intraperitoneal route. Immunization via conjunctiva resulted in 33% survival of C57BL/6 mice that received TTd adjuvanted with *B. pertussis*.
Conclusions: Conjunctival immunization induced TTd-specific local and systemic immune response, as well as a partial protection against systemic lethal challenge with tetanus toxin in C57BL/6 mice.

P1904
On the role of antigen stability in antigen cross-presentation

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Purpose/Objective: It has been previously shown that antigen (Ag) stability controls Ag presentation to $CD4^+$ helper T cells (Thai *et al.*, J Immunol., 2004) and thus influences antibody production (Delamarre *et al.*, J Exp Med., 2006). The aim of this study is to examine whether Ag stability also influences Ag cross-presentation.
Materials and methods: A model Ag containing the SIINFEKL $CD8^+$ T cell epitope covalently coupled to a 27-residues-long protein with three disulphide bonds was chemically synthesized, folded and purified by HPLC. A derivative differing by the absence of one disulphide bond was also synthesized. 3D structure was then assessed using circular dichroism. Susceptibility to proteolysis was examined using cathepsin L and cathepsin S. Susceptibility to reduction was examined using TCEP. The effect of Ag stability on cross-presentation was studied *in vitro* using two different approaches. First, the two derivatives were examined for their ability to trigger proliferation of splenocytes from transgenic mice expressing the SIINFEKL-specific TCR. Second, they were compared for their ability to activate a SIINFEKL-specific T cell hybridoma after cross-presentation by the JAWS II dendritic cell line.
Results: Circular dichroism experiments indicate that the two derivatives share the same overall structure. However, they differ in their susceptibility to proteolysis and to reduction. Thus, the three disulphide bonds containing protein is less susceptible to enzymatic degradation and to TCEP reduction than the derivative with the disulphide deletion. Differences were also found when cross-presentation was examined. Thus, the three disulphide bonds containing protein stimulates T-cells ten times less efficiently than the derivative with the disulphide deletion.
Conclusions: Our results show that two proteins differing by only one disulphide bond don't have the same cross-presenting capacity; the less stable one being more efficient than the more stable one. These preliminary results suggest that the stabilizing interaction network of proteins might modulate CD8 T-cell stimulation.

P1905
Prediction and identification of the potential immunodominant epitopes of herpes simplex virus type II glycoprotein D

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Purpose/Objective: This study aimed to determine the immunodominant epitopes (B cell epitopes) of herpes simplex virus II (HSV-2) glycoprotein D (gD) via software algorithms and experiments.

Materials and methods: B cell candidate epitopes of gD2 were predicted with the software DNAstar, Biosun, and Antheprot. Their three-dimensional structures were predicted by moe2008 software . The predicted peptide epitopes of gD2 were synthesized chemically. Subsequently, the antigenicity and immunogenicity of these peptide epitopes were examined by EIA and Western blot experiments. All conjugates of these peptides with the carrier protein BSA were used to immunize mice. The specificity of their antisera were investigated by EIA and Western blot. The ability to neutralize HSV-2 infectivity *in vitro* was investigated by a 50% plaque reduction assay on antiserum raised against each peptide.
Results: Four B cell candidate epitopes of gD,2 namely, peptides 6−18 (DPSLKMADPNRF), 223−232 (FIPENQRTVA), 255−267 (PELSDTTNATQPE), and 276−287 (ALLEDPAGTVS) of gD2 (1−290), were predicted and selected. The selected peptides have antigenicity and could react with the antibodies to gD2 (1−290). All conjugates of these peptides with the carrier protein BSA could induce mice to produce specific antibodies. The EIA and Western blot results showed that the antibodies against each of the four peptides reacted with the parent gD2 (1−290) and can neutralize HSV-2 infection *in vitro*. The antisera against peptide 6−18 of gD2 (1−290) showed the strongest neutralization activity, which has not been reported until now.
Conclusions: The immunodominant epitopes of gD2 screened by software algorithms and validated by experiments have good antigenicity and immunogenicity, may be used in viral diagnosis and vaccine design against HSV-2.

P1906

Probiotic bacterium, *Lactobacillus gasseri* SBT205,5 can enhance antigen-specific immune responses in oral mucosa

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Purpose/Objective: Probiotics are normal inhabitants of the gastrointestinal tract of man and are widely considered to exert a number of beneficial effects in many diseases. Probiotic strains have been used because of their potential therapeutic effects, and have been studied extensively. However, the mechanism by which they modulate the immune system is poorly understood. Periodontal disease has been suggested as a risk factor for systemic diseases. If the cascade of harmful immuno-inflammatory reactions could be reduced by the use of probiotics, probiotics could be have the potential to bring substantial health benefits for humans. In this study, we assessed the adjuvant effect of a probiotic strain, *Lactobacillus gasseri* SBT2055 (*L. gasseri*) for antigen-specific immune response.
Materials and methods: *Lactobacillus gasseri* were gastrically administered to 7-weeks-old BALB/c mice for 5 weeks. Three weeks after the initial administration, mice were immunized sublingually with 40-kDa outer membrane protein (40 k-OMP) of *Porphyromonas gingivalis* plus cholera toxin as mucosal adjuvant once a week for 2 weeks. Seven days after the last immunization, plasma, saliva, and nasal washes (NWs) were collected, subjected to ELISA and observed antibody (Ab) titer.
Results: Interestingly, *L. gasseri* treatment elicited significant increased levels of 40 k-OMP-specific S-IgA Ab in saliva and NWs. In addition, significant increased levels of 40 k-OMP-Specific IgG Ab were detected in plasma of these mice.
Conclusions: These results suggest that the administration of *L. gasseri* to the gastrointestinal tracts can induce the mucosal immune responses not only in the gastrointestinal tract but also in the oral cavity, which may have clinical implications for preventive or therapy of periodontal diseases.

P1907

Prophylactic treatment with IgY antibodies against influenza viruses does not interfere with the normal development of adaptive immunological memory against influenza

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Purpose/Objective: To develop new forms of intranasal-oral immuno-therapy by using IgY antibodies to control seasonal and pandemicinfluenza.
Materials and methods: Laying hens were immunized into the breast muscle with the high pathogenicity avian influenza (HPAI) H5N1 inactivated influenza viruses emulsified in Freund's complete adjuvant. After 4 weeks they were given a second injection with viruses in Freund's incomplete adjuvant. Eggs were collected commencing 2 weeks post second immunization and IgY antibodies were extracted from one volume of pooled egg yolks with nine volumes of super Q water. Balb/c mice were treated intranasally with 50 μg of IgY antibodies plus different concentrations of influenza viruses and were scored for the changes of weight as a sign if disease.
Results: Intranasal administration of specific IgY to mice protected against lethal infection of H5N1 avian influenza virus in 100% of the animals if administered at the same time as or 1 h prior to viral challenge. Interestingly, IgY against H5N1 also blocked viral invasion by H1N1 PR8 influenza virus during *in vitro* and *in vivo* challenges, demonstrating that IgY to H5N1 can indeed cross protect against infection with H1N1.

When mice, initially protected by IgY antibodies against H5N1, 3 months later were re-infected with a 100 times higher dose of H1N1 PR8 virus no reduction of weight was observed indicating the development of a good immunological memory to influenza viruses in these animals. *In vitro* challenge of immune T cells from such mice with antigen presenting cells, APCs, from infected lung showed that different populations of lung APCs induced different patterns of pro-inflammatory IFN-γ and IL-17. Also, influenza infected lung cells promoted TH cell independent proliferation of specific $CD8^+$ memory T cells.
Conclusions: The heterogeneity of the IgY response to viruses in chickens generates antibodies with broadly protective activity against influenza viruses and prophylactic treatment with IgY anti-influenza can be used to control influenza viral infection without interfering with the development of adaptive immunological memory resulting from a quiescent infection or a conventional vaccination.

P1909

Scientific investigations of trivalent influenza vaccine-induced adverse events in the paediatric population: role of innate immunity

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Purpose/Objective: During the 2010 Southern Hemisphere (SH) influenza season, there was an unexpected increase in the number of febrile reactions reported in the paediatric population (<5 years) in Australia shortly after vaccination with the CSL 2010 SH Trivalent Influenza Vaccine (TIV) compared to previous seasons CSL TIVs or other licensed 2010 SH TIVs. The rapid onset was suggestive of an innate immune response to the CSL 2010 SH TIV. A series of scientific investigations were initiated to identify the root cause of these adverse events (AEs). The primary objectives were: (1) investigate the immunogenicity/reactogenicity of the CSL 2010 SH TIV as compared to

previous season and comparator vaccines; (2) Identify vaccine components that may have contributed to the AEs; (3) identify surrogate parameters that can be used to prepare future TIVs which are safer and more effective in the paediatric population.
Materials and methods: *In vitro* cytokine/chemokine assays following stimulation of adult and paediatric whole blood, as well as mammalian cell lines such as the HEK293 cell based NF-κB reporter assay and various Toll-like receptor (TLR) transfectants, profiling of molecular signatures using microarrays, and *in vivo* studies in rabbits, ferrets, new born rats and rhesus non-human primates (NHPs).
Results: Various TIVs (approved commercial TIVs as well as re-engineered TIVs) and their individual monovalent pool harvest (MPH) components were examined. Detailed investigations eliminated bacterial-derived pyrogens and the method of inactivation. In addition, the role of neuraminidase was not found to be a significant contributing factor. The various *in vivo* and *in vitro* analyses indicated that the CSL 2010 SH TIV was, generally, more stimulatory and induced more potent gene signatures than previous seasons CSL TIVs or comparator 2010 SH TIVs. The *in vitro* assays also identified the B/Brisbane/60/2008 strain (and to a lesser degree the H1N1 pandemic A/California/07/2009 strain) to induce cytokines/chemokines and NF-kB activation. Interestingly, the induction of these signals was associated with a heat labile, viral-derived component.
Conclusions: The current working hypothesis is that the increase in febrile AEs reported in Australia after vaccination with the CSL 2010 SH TIV may be due to a combination of both the introduction of three entirely new strains in the CSL 2010 SH TIV, and differences in the manufacturing processes used to manufacture CSL TIVs compared to other licensed TIVs on the market.

P1910
Single step spray-dried polyelectrolyte microparticles enhance the antigen cross-presentation capacity of porcine dendritic cells

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Purpose/Objective: Vaccination is regarded as the most efficient and cost-effective way to prevent infectious diseases. Vaccine design nowadays focuses on the implementation of safer recombinant subunit vaccines. However, these recombinant subunit antigens are often poor immunogens and several strategies are currently under investigation to enhance their immunogenicity. The encapsulation of the antigens in biodegradable microparticulate delivery systems seems a promising strategy to boost their immunogenicity. Here, we evaluate the capacity of polyelectrolyte microparticles (PEMs), fabricated by single step spray-drying, to deliver antigens to porcine dendritic cells and how these particles affect the functional maturation of dendritic cells (DCs).
Materials and methods: PEMs were loaded with either BSA-FITC or F4 fimbriae, a bacterial adhesin purified from a porcine-specific enterotoxigenic *E. coli* strain, by co-spray-drying with the PEM constituents, the polyelectrolytes dextran-sulphate and poly-L-arginine and the sacrificial template mannitol. *In vitro* generated porcine monocyte-derived dendritic cells were incubated with these PEMs and their ability to internalise antigen-loaded PEMs and their phenotypical and functional DC maturation was assessed by confocal and live cell imaging, flow cytometry, proliferation assays and cytokine ELISAs.
Results: In confocal images we detected multiple particles per cell in >80% of the examined DCs, indicating that the resulting antigen-loaded PEMs were efficiently internalised by porcine monocyte-derived DCs. F4 fimbriae-loaded PEMs (F4-PEMs) enhanced CD40 and CD25 surface expression by DCs and this phenotypical maturation correlated with an increased secretion of IL-6 and IL-1β. More importantly, F4-PEMs enhance both the T cell stimulatory and antigen presentation capacity of DCs. Moreover, PEMs efficiently promoted the $CD8^+$ T cell stimulatory capacity of dendritic cells, indicating an enhanced ability to cross-present the encapsulated antigens.
Conclusions: Our results confirm recent data obtained in rodent models that single step spray-dried PEMs boost the immunogenicity of vaccine antigens and could accelerate the development of veterinary and human subunit vaccines based on polyelectrolyte microparticulate delivery systems to protect against a variety of extra- and intracellular pathogens.

P1911
T cell epitope distribution in viruses reveals patterns of protein biosynthesis

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Purpose/Objective: Development of T cell epitope vaccines is handicapped by the cost and difficulty associated with T cell epitope identification. Therefore, there is need for defining strategies that can speed translational vaccine research. Here, we tried to define a system for prioritizing protein antigens for vaccine design by investigating epitope distribution patterns.
Materials and methods: We used χ^2-statistics to analyze whether known CD8 T cell epitopes of Hepatitis C Virus, Human Immunodeficiency Virus-1 and Influenza A virus are distributed in the viral proteomes according to the size/length of the source proteins. We also analyzed the distribution of peptides predicted to bind to several human MHC I molecules using χ^2-statistics. Finally, we investigated the correlation between epitope distribution and sequence conservation.
Results: We found that epitopes are not distributed homogeneously by the size of the source proteins in any of the viruses. Specifically, structural proteins pack significantly more epitopes than those expected by their size. Moreover, we showed that such non-homogeneous distribution cannot be accounted by underlying MHC I-peptide binding preferences nor it is related to sequence conservation.
Conclusions: Overall, these results support the prioritization of structural antigens for epitope identification and vaccine design.

P1912
Targeting monocytes using a novel liposome based delivery system

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Purpose/Objective: Targeting specific cells of the immune system is a promising approach to improve the therapeutic index of immune modulating compounds. Monocytes play essential roles in inflammatory conditions in both systemic and local inflammation. Furthermore, monocytes can upon stimulation by innate activation of inflammatory cytokines differentiate into potent antigen presenting cells like macrophages and dendritic cells. The purpose of the current project is to target liposomes specifically to monocytes and thereby modulate their activation, differentiation and migration in inflammation and vaccine development.
Materials and methods: Liposomes with different biophysical properties were generated using phospholipids and modified lipids. Liposomes were prepared using the lipid film hydration method and extruded to reach an average size of 100 nm. Liposomes containing the fluorescent marker rhodamine were incubated with human PBMCs

and also used in whole blood assays, and the immune cell subsets were subsequently analysed for liposome association using FACS analyses.
Results: One of the liposome compositions tested showed a marked association with CD14 positive monocytes after only 15 min incubation in whole blood, whereas other types of liposomes associated with both monocytes and B-cells. The monocyte association was dependent on the age of the whole blood, indicating that factors in the blood were required for an efficient specific targeting process.
Conclusions: A specific liposome formulation showed strong association with CD14 positive monocytes after incubation in whole fresh blood for only 15 min. The targeting technologyis currently being explored further for suppression of monocyte function in relation to systemic and local inflammation, as well as for targeting to monocytes *in vivo*, with the aim of stimulating monocyte differentiation into antigen presenting dendritic cells able to raise an antigen specific immune response towards cancer or infectious antigens.

P1913
The effect of antioxidants on the development of antigen presentation cells

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Purpose/Objective: This study attempts to investigate the effect of reactive oxygen species (ROS) on the differentiation of the antigen presenting cells, including dendritic cells and polymorphonuclear leukocytes (PMN).
Materials and methods: The *in vitro* experiments were carried out by co-culturing the dendritic cells (DCs), the most potent antigen presenting cells, or the Gr-1+ cells from the bone marrow of C57BL/6J mice, with the L121-vaccine adjuvant-treated EL4 cells in the present of antioxidants, including catalase, tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), and butylated hydroxyanisole (BHA), followed by analysis of the expression of the costimulatory molecules in the DCs. The production of ROS in the cells was detected by 2',7'-dichlorofluorescene diacetate (DCFDA) and hydroethidine (HE). The *in vivo* experiments were performed in the C57BL/6 mice by immunizing the animals with the vaccine adjuvants containing ovalbumin (OVA), with or without pretreatment with the antioxidants. Animals were sacrificed on day 10 after immunization, and cells from bone marrow and spleen were isolated. The expression of the costimulatory molecules in DCs were determined by staining the cells with flurochrome-conjugated antibodies, followed by flow cytometric analysis.
Results: Our results showed that treatment with antioxidants reduced the MHC II expression and the production of H_2O_2 and $O_2ø$ in the CD11c+ splenic DCs both *in vitro* and in the L121-vaccine adjuvant treated animals. The population of the SSC-hi, Gr-1(+) cells in bone marrow in the vaccinated animals, pretreated with the antioxidants, was elevated at the expense of the lineage-2 (+) populations, accompanied by a significant reduction in the production of reactive oxygen species.
Conclusions: Data obtained in this study showed the potential roles of the reactive oxygen species on the differentiation of myeloid antigen presenting cells in the vaccinated animals.

P1915
The effect of vaccination with recombinant *L. donovani* and *L. mexicana* gamma glutamyl cysteine synthetase by different routes of administration on host immune responses

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Purpose/Objective: Human leishmaniasis is a spectrum of diseases caused by protozoan parasites of the genus *Leishmania*. Drugs existing for the treatment of leishmaniasis have been unsatisfactory. Ideally a vaccine could prevent infection and would be a feasible control method as infected individuals are resistant to clinical re-infection. In this study the ability of recombinant *L. donovani* and *L. mexicana* gamma glutamyl cysteine synthetase (γGCS) to induce a protective immune response in BALB/c mice was determined.
Materials and methods: Animals were immunised by different routes with the recombinant proteins and the effect of vaccination on production of parasite-specific IgG1 and IgG2a was determined using an ELISA assay. In addition the effect of immunisation on cytokine production by *in vitro* stimulated splenocytes from immunised mice was determined.
Results: Immunization with g GCS inducing significantly higher titers ($P < 0.05$) comparing to control group.
Conclusions: Vaccination induced significant Th1 and Th2 immune responses after single dose treatment.

P1916
The nature of protective immune response elicited by fructose 1, 6-bisphosphate aldolase against *Streptococcus pneumoniae* in mice

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Purpose/Objective: Fructose Bisphosphate Aldolase (FBA) was previously shown to be surface-localized pneumococcal adhesin with age-dependent immunogenicity in children and recombinant FBA (rFBA) elicited protective immune response against *Streptococcus pneumoniae* in mice. To evaluate the nature of the protective immune response, the cytokine profile elicited by rFBA immunization was explored.
Materials and methods: BALB/c mice were immunized with rFBA in the presence of CFA and IFA in booster immunizations. $CD4^+$ T-cells, obtained from rFBA-immunized mice, were co-cultured with rFBA-treated naïve antigen presenting cells. $CD4^+$ T-cells proliferation and cytokine profile was determined by ELISA. Survival was monitored daily following inoculation of mice with bacteria pre-incubated with antiserum against rFBA.
Results: Immunization with rFBA resulted in significant $CD4^+$ T-cells proliferation and cytokine secretion. IFNγ and IL-17 levels increased gradually and peaked at 72 h (h), while IL-2 level peaked at 12 h and declined thereafter. IL-4, IL-5, IL-10, IL-12p70 and TNFα levels peaked at 48 h and decreased or maintain their level at 72 h. In addition, antibodies to rFBA, IgG1 and IgG2A subtypes, neutralized bacterial virulence and prolonged the survival of mice.
Conclusions: These results demonstrate the involvement of Th1/Th2/Th17 family of cytokines in the protective immune response elicited by rFBA to *S. pneumoniae*.

P1917

Transcriptional response to ISCOM-Matrix at the site of administration and in the draining lymph node in pigs

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Purpose/Objective: ISCOM vaccines induce a balanced Th1/Th2 response, long-lasting antibody responses and cytotoxic T lymphocytes. Accordingly, in a recent human phase I study of influenza vaccination, the ISCOM-Matrix (Matrix M™) increased both the humoral and T cell-specific responses while still being well tolerated. The mechanism of action for ISCOM-Matrix is still largely unknown, thus the early immune response to ISCOM-Matrix was evaluated *in vivo*. The pig was chosen for the study due to immunological similarities to man, such as the organisation of monocytes, macrophages and DCs into subpopulations. Also the distribution of TLRs and the type I IFN response in the pig resemble those in human.
Materials and methods: Pigs were intramuscularly injected with 150 mg ISCOM-Matrix (Matrix M™) and sacrificed after 24 h. The Affymetrix GeneChip Porcine Genome Array was used to measure the early transcriptional response at the injection site and in the draining lymph node.
Results: After ISCOM-Matrix treatment, 594 genes at the injection site and 362 genes in the lymph node were differentially expressed (fold change >2; $q < 0.05$). Gene-set enrichment analysis revealed interferon-regulated genes (IRGs) to be significantly enriched in both tissues analysed, and at the injection site a gene signature for the cell type 'plasmacytoid dendritic cells' was enriched. In the draining lymph node, more than 40% of the up-regulated genes were IRGs. Of the 76 IRGs up-regulated, only *CASP1* and *DUSP5* were up-regulated in both tissues. Furthermore, genes encoding the cytokines osteopontin (*SPP1*), IL-10 and IL-18 were up-regulated at the injection site, and in the draining lymph node IL-1β was up-regulated but IL-18 was down-regulated.
Conclusions: The gene signature induced by ISCOM-Matrix was strongly characterised by an interferon-related response, although notably different IRGs were expressed at the injection site and in the draining lymph node. Elucidating these different responses might provide insights into the mechanism of action for the ISCOM-Matrix and its immunomodulatory effects. The pig will be a useful experimental tool in these further studies.

P1918

Understanding and manipulating chemokines and their receptors to improve vaccine adjuvant design

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Purpose/Objective: New and improved adjuvants are essential for the development of novel vaccines. However, we have a very limited understanding of how adjuvants work *in vivo*, even for adjuvants in widespread clinical use, such as Alum. Our previous work has shown that the injection site responses to Alum are required for adjuvant activity, although this requirement is transient and becomes dispensable within 2 h of administration. We therefore characterized the cells and molecules of the inflammatory response induced at the site of Alum injection during this timeframe.
Materials and methods: Chemokine and chemokine receptor expression at the site of alum injection was determined using Taqman® Low Density Arrays. The effect of alum on cell recruitment to injection sites was determined using flow cytometry.
Results: Alum injection was found to induce expression of several inflammatory chemokines, including CXCL1, CXCL2, CCL5 and the chemokine receptor CCR5 within 1−2 h of administration. Consistent with this expression pattern, *in vivo* phenotyping of the cells recruited to the site of injection within 2 h of Alum administration revealed a significant early influx of neutrophils compared with controls. Our studies are now focussing on the role of neutrophils in mediating the immune response to alum and the potential to alter or enhance the effects of alum through chemokine and chemokine receptor modulation. Specifically, we aim to determine the impact chemokines and their receptors have on antigen presenting cell function and subsequent T and B-cell responses.
Conclusions: As chemokines and their receptors are one of the key regulators of immune cell movement and immune responses, manipulation of these molecules represents a potential mechanism for altering or enhancing adjuvant function and the subsequent adaptive immune response.

P1919

Vaccine development in prion diseases

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Purpose/Objective: Prion diseases are fatal neurodegenerative disorders caused by the abberant accumulation of misfolded cellular prion protein (PrP^C) conformers, denoted as infectious scrapie isoform PrP^{Sc}. B- and T-cell tolerance are the main obstacles for an effective immune response to the incorrectly folded self-protein or to PrP of other mammals. The development of a potential anti-TSE passive or active vaccine thus represent a major problem. The possible ways of active and passive vaccine will be discussed.
Materials and methods: Three synthetic peptides, composed of 13 amino acids were chosen from the primary structure of human PrP and covalently bound to KLH in order to provoke the immune response in BALB/c mice. Monoclonal antibodies (mAbs) were produced by hybridoma technology and after stringent selection, one of them was chosen for humanisation which was done by using variable domain resurfacing approach guided by computer modelling. Mouse and humanized single-chain antibody fragments were expressed in bacterial cells using pMD204 expression vector. In futher studies, it was linked to apeptide, which enables crossing the blood-brain barrier. Different ways to induce anti-idiotypic responses were studied in xenogenic, alogenic and syngenic experimetal systems.
Results: PrP^{Sc}-specific IgG mAb was prepared, which recognizes a new, recently discovered fragment in TSE infected brain, PrP226*, which can be detected without PK digestion of brain tissue and could represent a form of PrP^{sen}. To examine a passive vaccine, scFv fragment of PrP^{Sc}-specific mAb was prepared in humanised form and it was linked to a peptide which enables crossing of the blood-brain barrier. As a new challenge in prion research, anti-idiotypic mAbs were prepared as a possible way to exercise an active vaccine, by using a panel of mAbs for stringent selection and an overview of the immunogenicity of the prion peptides was done.
Conclusions: In our studies, we were able to show that anti-idiotypic antibodies can be induced by careful selection of the antigen and the screening procedure. Besides, humanised scFv fragments of monoclonal antibodies, linked to a peptide, can cross the blood-brain barrier and can also be a model for the treatment of other neurodegenerative

diseases. The application of various epitopes, represented by a mixture of PrP peptides, or by a multivalent vaccine, should also be considered in future studies.

P1920

WHV preS1 elicits protective and virus-neutralizing antibodies in woodchucks

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Purpose/Objective: Chronic Hepatitis B virus (HBV) infection remains a serious health threat leading to cirrhosis and hepatocellular carcinoma. Current safe and effective prophylactic HBV vaccines consist of recombinant particles built by the small HBV envelope protein (S-HBs). They successfully reduced HBV related morbidities. However, these types of vaccines do not elicit an immune response in 5% to 10% of normal recipients. In addition, vaccine-induced immune escape mutants have frequently been described. Recently it has been shown that the preS1 domain of the large HBV surface protein (L-HBs) is involved in the initial binding, fusion and entry into hepatocytes and possesses HBV neutralizing epitopes. The ongoing debates about additional protective effect of preS1 domain are limiting the usage of promising preS1-containing third generation HBV vaccines.

Woodchucks are the natural host of woodchuck hepatitis virus (WHV) and are frequently used animal models to study the infection course, treatment and vaccination against Hepadnaviral infections. In present study we examined the capacity of synthetic WHV preS1-derived peptides to elicit protective antibodies against subsequent WHV infection and to neutralize infection *in vitro*.

Materials and methods: Three groups of naïve woodchucks were immunized with preS1-GST protein, myristoylated or non-myristoylated preS1 peptides. Animals were inoculated intravenously with 10^6 WHV genome equivalents 2 weeks after the last immunization and monitored for markers of infection. Neutralizing capacity of α-preS1 specific antibodies was tested in *in vitro* Hepatitis D virus (HDV) neutralization assay.

Results: Immunization with all synthetic WHV preS1-derived sequences protected woodchucks from WHV infection and α-preS1 specific antibodies efficiently neutralized HDV infection of primary woodchuck hepatocytes. The neutralization capacity of α-preS1 antibodies was depended on peptides modification. The natural *N*-terminal myristoylation of preS1 reduced the protective effect most likely due to sterical shielding of important epitopes.

Conclusions: The results demonstrated that the α-preS1 specific antibodies are neutralizing and protective. The third generation HBV vaccines containing preS1 domain should be regarded as the HBV vaccine of choice.