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Retinoic Acid Modulates the Early Expansion and Differentiation of CD4(+) T Cells During the Development of Intestinal Inflammation

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Published in:
Scandinavian Journal of Immunology

Publication date:
2014

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Rivollier, A. M. C., Pool, L., Wendland, K., & Agace, W. W. (2014). Retinoic Acid Modulates the Early Expansion and Differentiation of CD4(+) T Cells During the Development of Intestinal Inflammation. *Scandinavian Journal of Immunology*, *79*(6), 440-440.

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Abstracts

Scandinavian Society for Immunology
42nd Annual Meeting
Reykjavik, Iceland, June 11–14, 2014

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SCANDINAVIAN JOURNAL OF Immunology
AN INTERNATIONAL MONTHLY JOURNAL

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Workshop 1. Innate Immunity and Inflammation

WS1.1

Granulocyte Reactivity is Associated with Cartilage Destruction in Patients with Anti-collagen Antibodies

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Objective: RA patients with anticollagen antibodies (anti-CII) are characterized by acute RA onset and early joint erosions as well as increased production of TNF by peripheral blood mononuclear cells (PBMC) stimulated by anti-CII immune complexes (IC) *in vitro*. The aim was to investigate whether polymorphonuclear neutrophil granulocytes (PMN) react towards anti-CII IC and whether such responses are associated with the clinical acute-onset RA phenotype.
Methods: A set of 72 baseline patient sera (24 anti-CII positive, 24 anti-CII negative/RF positive and 24 anti-CII negative/RF negative) was chosen from a clinically well-characterized RA cohort with 2-year radiological follow-up (Larsen score). PMN and PBMC isolated from healthy donors were stimulated with anti-CII IC prepared with sera from the patients. PMN expression of CD11b, CD66b, CD16 and CD32 was measured by flow cytometry, whereas PMN production of myeloperoxidase (MPO) and IL-17, and PBMC production of TNF, was measured with ELISA.
Results: CD11b, CD66b and MPO were upregulated, whereas CD16 and CD32 were downregulated by anti-CII IC. There were correlations between CD16, CD66b and MPO production in relation to anti-CII levels ($r = -0.3152$, 0.6755 and 0.2532, respectively). CD16 was associated with early joint erosion ($P = 0.024, 0.034, 0.046$ at baseline, 1 and 2 years), and CD66b was associated with changes in joint erosion ($P = 0.017$ and 0.016, at 1 and 2 years compared to baseline, respectively). CD66b was associated with baseline CRP and PBMC production of TNF was associated with baseline ESR, in accordance with our earlier findings. No clinical associations were observed for MPO or IL-17.
Conclusion: PMN reactivity against anti-CII IC is associated with Larsen score and may contribute to joint destruction in newly diagnosed RA patients with high levels of anti-CII.

WS1.2

Increased IRAK-M in Peritoneal Macrophages of NOD Mice Suggests In Vivo Exposure to Bacterial Endotoxins from the Gut

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Macrophages play an important role in innate immune reactions, and peritoneal macrophages guard the sterility of this compartment mainly against microbial threat from the gut. Type-1 diabetes (T1D) is an autoimmune disease in which gut microbiota and gut immune system appear to contribute to disease pathogenesis. We have observed increased permeability and free radical production of gut epithelium in non-obese diabetic (NOD) mice. Impaired barrier function could lead to bacterial endotoxin leakage to peritoneal cavity. To seek for extraintestinal consequences of impaired barrier function, we characterized peritoneal lavage cells from young NOD mice after weaning. We detected a rapid increase in the number of macrophages in 1–2 weeks from weaning in NOD mice compared to C57BL/6 and BALB/c mice, and to NOD mice fed an antidiabetogenic diet (ProSobee) which improves gut barrier function. Upon LPS stimulation ex vivo, macrophages produced low amounts of TNF-α and showed reduced expression of CD40. Expression of TLR signalling pathway negative regulator, interleukin-1-associated kinase-M (IRAK-M), was increased, indicating prior *in vivo* exposure to TLR-4 ligand(s). Upon further phenotyping, increased levels of ARG1 were observed suggesting *in vivo* differentiation towards M2-like phenotype. Bacterial endotoxins in peritoneal cavity may contribute to autoreactive T cell activation by increased production of cytokines and activation of antigen-presenting cells in pancreatic lymph nodes.

WS1.3

Roles for Microglia Subsets in Neuroinflammation

Agnieszka Wlodarczyk, Morten Løbner, Oriane Cédile & Trevor Owens

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Experimental autoimmune encephalomyelitis (EAE) is a mouse model for the inflammatory demyelinating disease – multiple sclerosis (MS). MS is considered to be mediated by autoreactive myelin-specific T cells. The crucial event in generating autoimmunity in the central nervous system (CNS) is presentation of autoantigen to naïve T cells in lymph nodes and their reactivation in the CNS by infiltrating or resident antigen-presenting cells (APC). Dendritic

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cells (DC) are the major APC in the periphery but in the CNS they likely contribute to control of T cell entry. Microglia are CNS-resident cells and are considered to act as APC.
In this work, we compared antigen-presenting capacity of $CD11c^+$ and $CD11c^-$ microglia subsets with infiltrating $CD11c^+$ APC, which include DC. Two subpopulations of microglia ($CD11c^-$ $CD45^{dim}$ $CD11b^+$) and ($CD11c^+$ $CD45^{dim}$ $CD11b^+$), as well as $CD11c^+$ $CD45^{high}$ cells, were sorted from CNS of C57BL/6 mice with EAE. Sorted cells were characterized by flow cytometry for surface phenotype and by qRT-PCR for cytokine expression. They were co-cultured with primed T cells to measure the induction of T cell proliferation and cytokine response.
Our results show that the number of $CD11c^+$ microglia cells significantly increases during EAE. This population of cells unlike $CD45^{high}$ $CD11c^+$ cells express almost no CCR2 and express CX3CR1. $CD11c^+$ microglia differ significantly from blood-derived $CD11c^+$ cells also in their cytokine profile, expressing no detectable IL-6, IL-12 or IL-23, low levels of IL-1β and high levels of IGF1. By contrast, $CD11c^-$ microglia express low but detectable levels of all these cytokines. Although CNS-resident and blood-derived $CD11c^+$ cells show equivalent ability to induce proliferation of primed T cells, $CD11c^+$ microglia induce lower levels of Th1 and Th17 cytokines.
Our findings show distinct subtypes of APC in the inflamed CNS, with a hierarchy of functional competence for the induction of T cell responses.

WS1.4

Notch Enhances the Endothelial Inflammatory Response

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Vascular endothelial cells (ECs) upregulate the Notch ligand Jagged1 when exposed to pro-inflammatory stimuli. We found that inhibition of Jagged1 or Notch1 in ECs impaired their inflammatory response to IL-1, including reduced expression of adhesion molecules, chemokines and the chemokine transcytosis receptor DARC. Furthermore, EC-targeted inducible knockout of the canonical Notch transcription factor *RBPJ/CSL* reduced inflammation in a mouse model of 2,4-dinitrofluorobenzene-induced contact dermatitis. A similar trend was seen when endothelial *JAG1* was deleted. Conversely, transgenic overexpression of active Notch1 in ECs enhanced the inflammatory response. In conclusion, we show for the first time that Notch signalling modulates gene expression in activated ECs, sharpening the inflammatory profile of the vasculature and giving it an edge towards leucocyte recruitment.

WS1.5

Type I Interferon Expressed by Glial Cells in the Central Nervous System Plays a Protective Role in EAE

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Interferon beta (IFN-b) is used to treat patients with multiple sclerosis (MS). IFN-b is a member of the type I IFN family, which share a common receptor (IFNAR), and is induced by stimulation of innate receptors. Type I IFNs are increased in the CNS during experimental autoimmune encephalomyelitis (EAE), an animal model for MS, and lack of IFN-b and type I IFN signalling worsens EAE, suggesting an important role for endogenous IFN-b in the CNS. The aim of this study was to identify the cellular source and role of endogenous type I IFN in the CNS. Mice were administered poly I:C into the cerebrospinal fluid via the cisterna magna and induction of IFN-b was demonstrated by *in vivo* imaging of a luciferase/IFN-b reporter mouse. Intrathecal administration of poly I:C transiently induced IFN-b in the brain and spinal cord, and microglia and astrocytes were implicated as cell source. EAE was induced in C57BL/6- and IFNAR-deficient mice by immunization with myelin oligodendrocyte glycoprotein 35–55. At onset of EAE, mice received a single intrathecal injection of poly I:C. This transiently prevented EAE progression. In contrast, intrathecal administration of poly I:C had no effect on progression of EAE in IFNAR-deficient mice, indicating type I IFN-mediated poly I:C-induced suppression of EAE. These results suggest that type I IFN induced within the CNS by glial cells plays a protective role in EAE. This underscores the role of endogenous type I IFN in mediating neuroprotection.

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WS1.6

Examination of Intestinal Eosinophil Phenotype and Function During Inflammation and Steady State

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The intestinal lamina propria contains relatively high numbers of eosinophils during steady state as well as inflammation. Eosinophil accumulation during inflammation is particularly pronounced during parasitic infections and certain inflammatory diseases of the gastrointestinal tract, such as eosinophilic oesophagitis and inflammatory bowel disease (IBD). Despite this fact, the function of gastrointestinal eosinophils remains largely unknown.

We have isolated and purified eosinophils from the large and small intestine of both mice and humans in healthy as well as in diseased states by MACS and FACS. Our preliminary data suggest that there is much more plasticity among eosinophils with regard to phenotype, localization and function than previously believed. Indeed, eosinophils appear to be localized to different anatomical compartments in patients with IBD, and express different surface receptors than their blood-borne counterparts. Thus, it appears that both mouse and human eosinophils are far more varied than the dogma portrays them to be.

WS1.7

IL-1β Stabilizes Human S100A9 by Ca^{2+}- and P38 MAPK-Dependent Phosphorylation

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S100A8 and S100A9 are cytosolic Ca^{2+}-binding proteins that are associated with acute and chronic inflammation and cancer. S100A9 predominantly forms heterodimers with S100A8, but there are data showing that S100A9 can also form homodimers. We have investigated the stability of human S100A8 and S100A9 proteins using transfected COS cells. Our data showed that, in single transfected cells, the S100A8 protein decayed within 24 h, while S100A9 was completely unstable. However, S100A9 protein expression could be rescued upon co-expression with S100A8 or inhibition of proteasomal activity. Interestingly, incubation of S100A9-transfected COS cells with proteasomal inhibitor or IL-1β led to the formation of protease-resistant S100A9 homodimers. It is known that IL-1β stimulation causes Ca^{2+} influx into the cells and we found that addition of a membrane-permeable Ca^{2+} chelator (BAPTA-AM) inhibited the stabilization of S100A9. Further, IL-1β stimulation can also induce the activation of the Ser/Thr kinase P38 MAPK, which can phosphorylate S100A9 at Thr-113. We could show that addition of the P38 MAPK inhibitor SB-203580 to IL-1β-stimulated cells reduced the stabilization of S100A9. Finally, using site-directed mutagenesis, we changed Thr-113 to Ala-113 and found that this mutant S100A9 protein could no longer be stabilized by IL-1β.

In summary, our data indicate that the human S100A9 protein can be stabilized by inflammatory stimuli that cause Ca^{2+}- and P38 MAPK-dependent phosphorylation of the protein. Because S100A9 is a ligand of TLR4, this mechanism may constitute an amplification step during an inflammatory reaction.

WS1.8

Understanding the LACC1 Biological Function and its Role in IBD Pathogenesis

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Introduction: Several GWA studies conducted in various populations have consistently identified *LACC1* as a Crohn's disease risk gene. *LACC1* (previously called chromosome 13 open reading frame 31, *C13orf31*) is located on chromosome 13q14.11, and its product consists of 430 amino acids and has a mass of 47.780 kDa. At present, its biological function is unknown as well as its pathogenic role in Crohn's disease. The fact that LACC1 predicted protein sequence is highly conserved across species calls for an important function; however, it is puzzling to observe that LACC1 homology is with laccase proteins that are uniquely found in bacteria.

Material and methods: For functional characterization of *LACC1*, HeLa, THP-1 and U937 cell lines were utilized. Additionally, immunofluorescence, Western blot and luciferase assay were conducted.

Results: From the real-time PCR and Western blot analyses, we identified that LACC1 was endogenously regulated in THP-1 and U937 cell line upon PMA-driven differentiation and particularly under LPS stimulation. The Western blot results indicated a cleavage of the protein when HeLa cells were transfected with the LACC1-wt construct and a N-terminus GFP-tagged protein. Interestingly, the immunofluorescence experiments on the THP-1 cells revealed partial co-localization with endomembrane structures and ER compartments. The luciferase assay conducted to investigate LACC1 involvement in unfolded protein response showed an increased luciferase activity in $LACC1^{+}$ versus $LACC1^{-}$ tunicamycin-treated HeLa cells.

Moreover, the laccase assays demonstrated that LACC1 is not a conventional laccase.

Conclusion: Preliminary experiments performed on LACC1 show possible co-localization of LACC1 in the endomembrane structures and its endogenous expression in macrophages; however, further experiments need to be designed in order to define its functionality.

WS1.9

The Salivary Scavenger and Agglutinin (SALSA) in Early Life: Diverse Roles in Amniotic Fluid and Intestine

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The salivary scavenger and agglutinin (SALSA), also known as gp340 and dmbt1, is produced by mucosal tissues throughout the body. SALSA is an antimicrobial and inflammation regulating molecule located at the mucosal surfaces. It has also been suggested to play a role in both cell differentiation and epithelial polarization. In the present study, a general screen of body secretions for SALSA revealed that SALSA was present in amniotic fluid (AF) and exceptionally abundant in both meconium and faeces of infants. SALSA constituted up to 6–10% of the total protein amount in meconium, thus making it one of the most abundant proteins. Innate immunity is extremely important for both the mother in the course of maintaining a healthy pregnancy and for the infant in the very early stages of life. The high levels of SALSA in AF, and especially in the meconium and faeces, suggest a robust and important function for SALSA both during the foetal development and in the innate immune defence of infants. SALSA proteins in the AF and intestinal samples were polymorphic and exhibited varying peptide compositions. In particular, a different abundance of peptides corresponding to functionally important structures was found in AF and intestinal SALSA. Peptides within the bacterial-binding part of the scavenger receptor cysteine-rich domains were more abundant in the intestinal SALSA. In contrast, SALSA present in the AF contained relatively more peptides from the zona pellucida (ZP) domain, which is involved in cell differentiation. Our results have thus identified body compartments, where SALSA is particularly abundant, and suggest that SALSA exhibits varying functions in the different locations.

WS1.10

CD11c/CD18 Phosphorylation on Alpha Chain Regulates Integrin Functions

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CD11c/CD18 (or complement receptor 4, αXβ2, or p150/95) is a leucocyte integrin expressed especially on monocytes, macrophages, dendritic cells as well as on some subsets of T and B cells. It binds to a variety of extracellular matrix molecules, soluble ligands and cell surface Ig-superfamily proteins. The broad spectrum of the binding partners is reflected on the functions of CD11c/CD18: one of its first reported functions was the function as a complement receptor, hence the name. Other suggested roles include dendritic cell antigen uptake and presentation, generating CD4 and CD8 T cell-mediated responses and providing a useful approach for antitumour vaccine development. Recently, CD11c/cD18 has also been shown to have a role in the transendothelial migration of macrophages to atherosclerotic plaques in hypercholesterolaemic mice. Yet another suggested role could be the removal of senescent red cells from the bloodstream through the interactions between red cell ICAM-4 and CD11c/CD18 on spleen macrophages.

The phosphorylation of the cytoplasmic parts of integrin α and β chains has been shown to be of pivotal importance in the regulation of the integrin activities like adhesion. Here, we identify the phosphorylation site of CD11c as serine 1158 and show that it is pivotal for adhesion and phagocytosis, but not for outside-in activation.

In general, it may be that the role of the CD11c/CD18 is relevant also outside the circulation, as it is enriched in macrophages and dendritic cells and binds to several extracellular matrix ligands. All the suggested functions involve adhesion, and understanding the regulation of CD11c/CD18 is a prerequisite for further elucidation of this complex integrin.

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WS1.11

Neutrophils from Patients with ANCA-Associated Vasculitis Exhibit an Increased Propensity to Undergo Neutrophil Extracellular Trap (NET) Formation and/or Necrosis

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Background: Dying neutrophils surrounding small vessels, the histological hallmark of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), may provide the source of antigens for the generation of ANCA. We have previously shown that AAV neutrophils are less prone to undergo cell death from apoptosis. Neutrophil extracellular traps (NETs), released in a pathway of programmed cell death called NET-osis, have recently been visualized at vasculitic lesions in patients and have also been shown to be important in AAV rodent model.
Methods: Neutrophils were isolated by Percoll centrifugation from 24 AAV patients (5 active/19 remission) and 15 healthy controls (HC). Neutrophils were exposed to 10 nM phorbol 12-myristate 13-acetate (PMA), 2 or 8 ng/ml TNF-α, 10 or 100 nM dexamethasone or left untreated. DNA was quantified in a microplate fluorescence reader using Sytox Green, and the results were expressed as percentage of total DNA (lysis with 1% Triton X-100). Neutrophil cell death was also visualized by fluorescence microscopy.
Results: PMA, also at moderate doses (10 nM), efficiently induced NET-osis but no difference was seen between neutrophils from AAV patients and HC. However, unstimulated neutrophils of AAV patients showed an increased propensity for cell death after 4 h compared to HC ($P < 0.05$). Stimulation of neutrophils with a high dose of TNF-α (8 ng/ml) abolished the difference in cell death between patients and controls, while a difference could still be detected after co-incubation with a low TNF-α dose (2 ng/ml). About 100 nM dexamethasone decreased PMA-induced NET-osis, while it increased spontaneous cell death.
Conclusions: Neutrophils from AAV patients show an increased propensity for spontaneous NET formation and/or necrosis *in vitro*. However, these differences can be abolished by the addition of inflammatory cytokines (i.e. TNF-α) or high doses of anti-inflammatory drugs. Further and more intricate studies are needed to reveal the significance of these findings.

WS1.12

Polysaccharide from *Achillea millefolium* has Anti-Inflammatory Effects in THP-1 Monocytes

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Background: *Achillea millefolium* has been used in traditional medicine to heal inflamed cuts and alleviate rheumatism. Aqueous extract from *Achillea millefolium* has been shown to have anti-inflammatory effects on dendritic cells *in vitro*. Several compounds have been isolated from *Achillea millefolium,* but only a few have been tested for their immunological effects.
Objectives: To use bioguided fractionation to isolate a polysaccharide from *Achillea millefolium* that has anti-inflammatory effects on THP-1 monocytes and to determine how the polysaccharide affects intracellular signalling in the cells.
Methods: Bioguided fractionation using ion-exchange chromatography was used to isolate a polysaccharide from *Achillea millefolium.* THP-1 monocytes were primed with IFN-γ and then stimulated with LPS in the absence or presence of the polysaccharide. The cells were cultured for 48 h, and cytokine concentration in the supernatant was determined by ELISA. Phosphorylation of several intracellular kinases will be determined by Western blotting after 1-h stimulation of the cells in the presence or absence of the polysaccharide.
Results: The water-soluble compound isolated from *Achillea millefolium* is most likely a polysaccharide with a molecular weight <20 kDa. THP-1 cells incubated with the polysaccharide increased IL-12 secretion by around 80% but increased IL-10 secretion by around threefold, thus decreasing the IL-12/IL-10 ratio around 40%. The effect of the polysaccharide on phosphorylation of intracellular kinases is currently being investigated.
Conclusions: A polysaccharide isolated from *Achillea millefolium* has anti-inflammatory effects on cytokine secretion by THP-1 monocytes.

WS1.13

Heightened Antibody Response to *Porphyromonas gingivalis* Detected in ACPA$^+$ RA, Suggesting a Role for this Oral Bacteria in the Aetiopathogenesis of RA

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Introduction: *Porphyromonas gingivalis* is a major cause of chronic periodontitis (PD) and the only known pathogen to express a peptidyl arginine deiminase (PAD) enzyme, therefore potentially contributing to the aetiopathogenesis of rheumatoid arthritis (RA), by triggering anticitrullinated protein antibody (ACPA) production. The overall aim of this study was to investigate whether *P. gingivalis* is an aetiological risk factor for the development of RA, specifically ACPA-positive RA, and specifically to determine whether ACPA$^+$ RA patients have an heightened immune response to *P. gingivalis*-specific virulence factor, arginine gingipain B (RgpB).

Methods: Sera from 66 patients with chronic PD, 60 non-PD controls, 1974 RA cases and 383 non-RA controls were screened by ELISA for the presence of antibodies to RgpB. For statistical analyses, Mann–Whitney *U*-test and an unconditional logistic regression analysis were used.

Results: Significantly higher anti-RgpB antibody levels were detected in the PD subset, compared to the non-PD controls. However, the correlation coefficient for being anti-RgpB IgG positive and having PD was weak ($r = 0.37$, $P < 0.001$). Significantly elevated anti-RgpB IgG levels were also detected in RA compared to non-RA controls, and in ACPA$^+$ RA compared to ACPA$^-$ RA. Regression analysis revealed significant associations between anti-RgpB IgG status and RA, with an OR of 3.3 for ACPA$^+$ RA and 2.4 for ACPA$^-$ RA. Moreover, there was an interaction between anti-RgpB IgG and smoking, and between anti-RgpB IgG and SE, in ACPA$^+$ RA, but not in ACPA$^-$ RA.

Conclusion: Anti-RgpB IgG should not be used as a surrogate marker for PD. However, our data suggest a role for the oral bacteria *P. gingivalis* in the aetiology of RA, because a heightened antibody response to RgpB was strongly associated with RA, specifically ACPA$^+$ RA, and because we could observe a (biological) interaction between anti-RgpB IgG and risk factors for RA, *HLA-DRB1* SE and smoking.

WS1.14

Increase in Acute-Phase Proteins in Early Life Predicts Reduced Growth Rate of Beef Calves at 6 Months of Age

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Parts of the innate immunity are activated after birth, manifested as elevated levels of the acute-phase proteins in clinically healthy animals for 2–3 weeks after birth. In the current study, we explored the use of the acute-phase response as a long-term prognostic marker for growth in beef calves.

Thirty-seven calves from six herds were visited three times at intervals of approximately 13 days, when calves were (mean ± S.D.) 3.3 ± 1.1, 16.4 ± 1.4 and 30.2 ± 1.2 days of age. On each visit, calves were clinically examined and weighed. Blood samples were collected and analysed for acute-phase proteins: haptoglobin, serum amyloid-A (SAA) and fibrinogen. Association between acute-phase proteins at different age groups and weight gain was explored by linear mixed models.

In general, the calves were healthy, and only sporadic health problems were detected. The haptoglobin concentrations over the reference value (obtained from samples of 120 healthy dairy calves) of 196 mg/l were observed six times, in five calves. Fibrinogen concentrations over the reference value of 5.9 g/l were observed on 20 measurements, in 12 calves. None of the calves had SAA concentration over the reference value of 178 mg/l. However, the linear mixed model showed that calves with higher SAA concentrations on the visit at approximately 2 weeks of age had lower growth rate from the first visit to the last weighing at 210 days of age.

Similar phenomenon at the second week of age – the innate immune response when no signs of inflammation can be observed associated with retarded growth in long term – has been detected in lambs and in reindeer calves (Orro *et al.* 2011, Comp Immunol Microbiol Infect Dis), but mechanisms for this interplay between growth and the activated innate immune system is yet to be solved.

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WS1.15

Periodontitis Enhances Inflammatory Cytokine Response in Rats with Pristane-Induced Arthritis

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There is growing evidence of a link between rheumatoid arthritis (RA) and periodontitis reflected in shared chronic nature of the inflammatory responses and a number of environmental factors including smoking and periodontal pathogens. Objectives: This study aimed to further understand mechanisms underlying the infection-immune association of periodontal niche with RA inflammatory events, related to the ability of the major periodontal pathogens to contribute to the loss of self-tolerance and development of RA. Methods: We examined Porfyromonas gingivalis and Fusobacterium nucleatum ligature-induced periodontitis and pristane-induced arthritis (PIA) interaction in rats by monitoring the disease progression and severity with clinical assessment, micro-CT and cytokine multiplex profiling at different time intervals after induction. Results: Experimentally induced periodontitis manifested clinically in all investigated subjects as severe localized form by week 6 following induction and resulted in significant alveolar bone loss that progressed further by week 15. Arthritis induction at week 8 was associated with a significant animal weight decrease followed by severe arthritic changes in all limbs. Endpoint analysis revealed a trend towards increased pro-inflammatory cytokine levels (IL-1b, IL-2, TNF-a and RANTES) in serum of periodontitis-challenged PIA rats. Conclusions: Periodontitis enhanced systemic inflammatory response in experimental arthritis in rats compared to their counterparts with no periodontal disease.

WS1.16

Comparison of the Ability of Clinical and Dairy Strains of Lactobacillus rhamnosus to Activate the Complement System

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Introduction: The complement system is a central part of innate immunity defending us against invading pathogens. *Lactobacillus rhamnosus* GG (LGG) is one of the most widely used probiotics in the world. However, several strains of *L. rhamnosus* have been isolated from various types of infections including bacteraemic sepsis. Earlier studies have found genomic and phenotypic differences between *L. rhamnosus* strains from various origins. The aim of this study was to analyse how different *L. rhamnosus* strains from clinical samples and dairy products activate or escape complement *in vitro*.

Methods: The clinical isolates of *L. rhamnosus* were collected at the Helsinki University Central Hospital laboratory. To study complement activation, *L. rhamnosus* strains were incubated with normal human serum at +37 °C for 30 min. Complement activation was analysed by measuring C3a and terminal pathway complex (TCC) formation by ELISA. The binding of complement regulators factor H and C4bp was analysed using a radioligand assay.

Results: All the studied strains activated complement as judged by the formation of C3a and TCC. The level of activation varied between the strains only to a limited extent. None of the *L. rhamnosus* strains bound complement inhibitors factor H or C4bp.

Conclusion: All tested strains of *L. rhamnosus*, including strain LGG, activated complement. No biologically relevant differences in complement-activating capacity or binding of the studied complement regulators were found between the *L. rhamnosus* strains from clinical or dairy origin.

WS1.17

The Human Cathelicidin LL-37 in the Skin of Individuals with Psoriasis

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Psoriasis is a common chronic skin disease associated with high degree of morbidity and low quality of life. Although a number of treatment options are available, psoriasis is linked to high cost and major impact on healthcare system and society. Induction of antimicrobial peptides and changes in proteolytic balance are characteristic of psoriatic skin. Furthermore, the human cathelicidin LL-37 has been linked to the pathogenesis of psoriasis, either as a trigger or by facilitating the maintenance of the disease. Therefore, understanding the role of this peptide of host defence can aid effective development of psoriasis treatment. Our previous data demonstrate that a controlled balneotherapy in the Blue Lagoon can suppress circulating and epidermal T lymphocytes, reduce skin inflammation and improve quality of life. Here, we hypothesize components of a combined balneo- and narrowband ultraviolet B (NB-UVB) therapy to positively affect the proteolytic imbalance and the high levels of antimicrobial and antiproteolytic peptides involved in the pathogenesis of psoriasis. In order to address this issue, we collected punch biopsies from psoriatic skin lesions before and after 6-week daily treatment involving bathing in geothermal seawater combined with NB-UVB therapy. Punches were sliced, and sections were fluorostained using an LL-37-specific antibody. Our results indicate that LL-37 levels are higher in samples collected from individuals after successful balneotherapy, particularly in the upper cell layers, that is, stratum granulosum and lucidum. In conclusion, the increased levels, stability and location of LL-37 after therapy are surprising, particularly because the location corresponds to the location of proteases, which degrade and inactivate LL-37. The data indicate an unknown homeostatic mechanism of LL-37 in the suppression of pathogenic skin infiltrating T effector cells.

WS1.18

Dendritic Cells Matured in the Presence of the Lycopodium Alkaloid Annotine Direct T Cell Responses Towards a Th2/Treg Phenotype

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Annotine is a lycopodane-type alkaloid that does not inhibit acetylcholinesterase, as some other lycopodium alkaloids do. It has not been investigated for other bioactivity. The aim of this study was to determine whether annotine affects immune responses by dendritic cells (DCs).

Annotine was isolated from Icelandic *Lycopodium annotinum* ssp. *alpestre*. Human monocytes were differentiated into immature DCs (imDCs) by incubating them with GM-CSF and IL-4 for 7 days. The imDCs were matured with TNF-α and IL-1β and stimulated with LPS for 48 h in the presence or absence of annotine at a concentration of 1, 10 or 100 μg/ml. The effect of the annotine on maturation of the DCs was determined by measuring concentration of cytokines in culture supernatant by ELISA and expression of surface markers by flow cytometry. DCs matured and stimulated in the absence or presence of annotine at 100 μg/ml were also co-cultured with allogeneic $CD4^+$ T cells for 6 days, and concentration of cytokines in supernatant was determined by ELISA.

DCs matured and stimulated in the presence of annotine secreted less IL-10, IL-12 and IL-23 than DCs matured and stimulated in the absence of annotine. However, annotine reduced IL-10 secretion more than IL-12 secretion, making the ratio of IL-12/IL-10 in medium from DCs cultured with annotine higher than that in medium from DCs cultured without annotine. Allogeneic $CD4^+$ T cells co-cultured with DCs matured and stimulated in the presence of annotine secreted more IL-10 and IL-13 than T cells co-cultured with DCs matured and stimulated in the absence of annotine.

Although annotine had a pro-inflammatory effect on cytokine secretion by DCs, DCs matured and stimulated in the presence of annotine had a greater potential to direct the differentiation of T cells into a Th2/Treg pathway than DCs matured and stimulated in the absence of annotine.

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WS1.19

Cytotoxic Peptides from S. aureus Cause Neutrophil Cell Death with NET-like Features

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Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) are antibiotic-resistant strains with high virulence that cause severe infections in humans. These strains secrete a group of virulence factors called phenol-soluble modulins (PSMs) which are peptides capable of activating neutrophils to produce reactive oxygen species (ROS) through the chemoattractant receptor FPR2, but also to possess cytotoxic properties at higher concentrations.

We found that PSM peptides (PSMα2 and PSMα3) were cytotoxic to isolated human neutrophils and moreover that the neutrophils formed neutrophil extracellular trap (NET)-like structures after treatment with the peptides. NET-like structures were not induced by other cytotoxic peptides (e.g. high doses of LL-37), and the PSM-triggered NET-like structures were morphologically indistinguishable from NETs induced by conventional stimuli, such as phorbol myristate acetate (PMA), in that they contained extracellular DNA, histones and granule proteins. The NET-like structures induced by PSM were also potently antifungal and capable of inhibiting *Candida albicans* growth *in vitro*. The cytotoxic effect of the PSM peptides was not mediated via FPR2 and in contrast to NET formation by PMA, the PSM-triggered process induced very rapid expulsion of the DNA structures (within minutes). NET formation by PMA has been shown to be dependent on ROS production, but the NET-like structures induced by PSM peptides were independent of radical formation.

Our data reveal an unusual cytotoxic effect by PSM peptides resulting in a type of cell death that morphologically resembles NETosis very closely. Thus, these findings are the basis for further studies on the role of these NET-like structures for the pathogenesis of CA-MRSA.

WS1.20

Boosting Innate Immunity with New Potent Inducers of Antimicrobial Peptide Expression

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The continual emergence of antibiotic resistance among bacterial pathogens poses a great challenge to the public health. The pipeline of new antibiotics in drug development has yet to match this threat, because only few novel agents have been developed in the last decades. Strengthening immune defences against pathogens by boosting the expression of our own 'natural antibiotics' may represent a novel or complementary pharmaceutical intervention in infectious diseases. Importantly, the multiplicity of antimicrobial peptides (AMPs) with overlapping antibacterial mechanisms secures minimal risk of microbial resistance.

AMPs can be induced by certain compounds, as exemplified by butyrate and 4-phenylbutyrate (PBA). In a rabbit model of shigellosis, oral administration of PBA has been shown to eliminate the pathogen in rectum and colon and recovery from the symptoms of the disease.

We here present a new class of compounds that are considerably more powerful inducers of the human AMP LL-37 (even at down to 800 times lower concentration compared to PBA). Furthermore, a synergistic effect is obtained when these compounds are used in combination with vitamin D. These results were demonstrated both in a reporter cell line containing a construct with luciferase coupled to the human *CAMP* gene encoding LL-37 (Nylén *et al.* 2013) and confirmed in the parental colonic epithelial cell line HT-29. The new class of compounds is aroylated phenylenediamines or derivatives or analogs thereof.

The effectiveness of this new class of inducers is unexpected and implies that these compounds may work *via* different or additional stimulatory mechanisms compared to previously identified inducers. Although some of the new inducers are known to be histone deacetylase (HDAC) inhibitors, no clear relationship between effectiveness (IC50) for HDAC inhibition and stimulation of LL-37 expression is found, suggesting that the induction occurs mainly through another mechanism.

WS1.21

Distribution of the 1637delC Allele Among MBL2 Genotypes

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Activation of complements via the lectin pathway (LP) is mediated by five separate pattern recognition proteins (PRPs). These are mannan-binding lectin (MBL), collectin-11 (CL-11), ficolin-1, ficolin-2 and ficolin-3. MBL insufficiency is associated with susceptibility to infectious diseases. The frequency of MBL insufficiency (defined as *AX/0* and *0/0* genotypes) has been determined to be 7.9% among Icelandic blood donors (IBD). This is relatively high among healthy individuals and suggests that MBL might be a redundant molecule. Ficolin-3 is the most abundant of the PRPs in serum. The *1637delC* mutation (allele frequency 0.01) in the *FCN3* gene causes ficolin-3 deficiency in a gene dose-dependent manner.

If MBL insufficiency is compensated for by, for example, ficolin-3, then it is conceivable that ficolin-3 levels in MBL insufficiency are higher than in sufficient cases. Therefore, we hypothesize that the *1637delC* allele should be rare or not found among *AX/0* and *0/0* genotypes. The aim was to investigate the distribution of the *1637delC* allele among *MBL2* genotypes.

The cohort consisted of blood donors and individuals that had been referred to our laboratory for MBL evaluations (n = 637). MBL deficiency variants in exon 1 (*B–D*) were determined in addition to the downregulating allele *X* in the *MBL2* promoter using melting curve analysis on the LightCycler. The *1637delC* allele was determined by RFLP-PCR.

The resulting *MBL2* genotypes were grouped into insufficient producers of MBL (*AX/0* and *0/0*; n = 106) and sufficient (*A/A* and *AY/0*; n = 531). Twenty *1637delC* heterozygotes were detected in the sufficient group (3.8%), whereas the allele was not found in the insufficient group (P = 0.0426).

The results support our hypothesis that MBL-insufficient individuals are not carriers of the *1637delC* allele. This could be explained by natural selection and reveals the importance of this defence system.

WS1.22

Bronchial Epithelial Model VA10 and Analyses of the Mechanism for Phenylbutyrate-Induced Defences

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Antimicrobial peptides (AMPs) form an important part of innate immunity in humans and constitute the first line of defensive barrier against the invading microbes preventing infections. In our current study, we looked at the effect of histone deacetylase inhibitor 4-phenyl butyric acid (PBA) on innate immune effectors and associated regulatory pathways in a human bronchial epithelial cell line VA10. PBA significantly induced expressions of several innate effectors pro-LL-37 (cathelicidin), DEFB1 (human beta defensin 1) and others in a time- and dose-dependent manner. In addition, PBA significantly enhanced Toll-like receptor 5 ligand flagellin-mediated pro-inflammatory cytokine expression. This activation was shown dependent on activation of MAP kinases. Further modulation of nuclear receptors differentially affected PBA induction of *CAMP* expression in an unexpected way. We also show the effect of PBA on enzymes of the vitamin D metabolic pathway affecting cathelicidin regulation. Antimicrobial peptides have been indicated as effectors of wound healing. TGFB1, EGFR and transcription factors of cell differentiation affected PBA-induced LL-37 expression. This indicates a cross-talk between pathways involved in growth and differentiation with innate immune regulation. Finally, we have shown PBA- and vitamin D_3-induced antibacterial activity. Our future goal is to understand detailed mechanism behind PBA-induced antimicrobial effectors identifying both cis and trans factors involved in regulation and ultimately strengthening the use of PBA as a therapeutic drug against infections.

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WS1.23

Frequency of Lectin Pathway Components Deficiency Alleles and Genotypes in Icelandic Blood Donors

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The lectin pathway (LP) of complement activation is initiated by five separate pattern recognition proteins (PRPs) that can either be mannan-binding lectin (MBL), ficolin (1–3) or collectin-11 (CL-11). Each of these PRPs is complexed with serine proteases (MASP-1–3) and non-enzymatic proteins (MAP1 and sMAP). Several mutations cause deficiency in the LP components. Individuals with the *MBL2* genotypes *0/0* or *XA/0* have been defined as MBL insufficient. The effect of the *D120G* allele in the *MASP2* gene and the *1637delC* allele in the *FCN3* gene is gene dose dependent and homozygotes have no detectable MASP-2 and ficolin-3 in serum.

Our aim was to evaluate the frequency of these deficiency alleles and genotypes in Icelandic blood donors.

EDTA blood samples were collected from 500 blood donors from the Icelandic Blood Bank and DNA isolated using high salting-out procedure. A multiplex *real-time PCR* assay with subsequent *melting curve analysis* was performed to detect variants in the *MBL2* gene (*X/A/B/C/D*). To detect the *1637delC* mutation, RFLP-PCR was performed. Genotyping for the *D120G* mutation was carried out using PCR-SSP.

A total of 498 blood donors were genotyped for the *1637delC* and *D120G* alleles. Fifteen (3%) individuals were found to be heterozygous for *1637delC* and 39 (7.8%) were found to be heterozygous for *D120G*. A total of 494 individuals were *MBL2* genotyped. The frequency of MBL-sufficient genotypes (*YA/YA, XA/XA* and *YA/0*) and MBL-insufficient genotypes (*XA/0* and *0/0*) was 92.1% and 7.9%, respectively.

Estimated allele frequencies of *1637delC* and *D120G* are 0.015 and 0.041, respectively. We can therefore expect approximately 1:4500 to be ficolin-3 deficient (~70 individuals) and 1:640 to be MASP-2 deficient (~500) in the Icelandic population. These results are comparable to population studies in other Caucasian populations. However, the frequency of MBL insufficiency is considerably lower than found elsewhere in European Caucasians (7.9% versus 16%).

WS1.24

The Effects of Carbamylated LL-37

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Background: Non-enzymatic conversion of lysine residues to homocitrulline (carbamylation) is a common post-translational modification (PTM) of proteins and peptides, which occurs in inflammatory foci. The process of lysine carbamylation is mediated by the myeloperoxidase (MPO) system ($MPO/H_2O_2/SCN^-$) employed by neutrophils to kill bacteria. Thus, MPO has recently attracted a lot of attention as a potential trigger factor for atherogenesis and inflammation.

Upon infection, immune cells and endothelial cells start to secrete the antimicrobial and immune modulatory peptide LL-37 in order to fight invading pathogens. Thus, LL-37 is mainly present in an inflammatory milieu where it is frequently exposed to carbamylation. In this context, the aim of our study was to analyse the effects of carbamylated LL-37 in the interaction with human and bacterial cells.

Materials and methods: Synthetic LL-37 peptides with lysine residues replaced with homocitrulline at sites that we found to be vulnerable to carbamylation were used. The bactericidal capacity of carbamylated LL-37 was investigated in liquid cultures of *B. subtilis*, *E. coli* and *S. aureus*. Transmigration assay was performed using 6.5-mm transwell plates with 3 μm pore size. PMNs and erythrocytes were used in lysing assays.

Results: Carbamylation has profound impact on LL-37 bactericidal and immunomodulatory activities. We found that carbamylation decreases bactericidal capacity of LL-37 against *B. subtilis* and *E. coli*. Interestingly, LL-37 carbamylated at positions 12 and 15 was strongly lytic to PMN, but had no effect on erythrocytes. Additionally, LL-37 with lysine carbamylated at positions 12 and 15 acted as strong chemoattractant for PMNs as compared to native peptide.

Conclusions: Taken together, our data suggest that carbamylation has a profound impact on the activity of the LL-37 and may be detrimental to the host. Modified LL-37 exerted strong pro-inflammatory activity that may lead to exacerbation of inflammation with concomitant collateral host tissue damage.

WS1.25

M2-Macrophages to Clear Amyloid Beta Oligomers in Alzheimer's Disease Mice

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In Alzheimer's disease (AD) transgenic mice, a pro-inflammatory activation of microglia/macrophages in the brain contributes to amyloid beta buildup and cognitive decline. Reversal of this effect, by switching of the microglial phenotype from pro-inflammatory (M1) to anti-inflammatory (M2), is associated with better clearance of amyloid beta deposits. In this study, we wanted to test the hypothesis that *in vitro*-generated M2 macrophages could phagocytose and clear amyloid beta in transgenic AD mice. We show that M1 and M2-activations have opposite effects on the ability of macrophages to phagocytose amyloid beta oligomers, with M2 increasing and M1 decreasing phagocytosis. Interestingly, when macrophages are transplanted into the hippocampus of aged transgenic AD mice, they retain the ability to migrate to plaques and phagocytose amyloid beta. Furthermore, we show that this beneficial response towards amyloid beta is associated with an upregulation of scavenger receptors, because both broad pharmacological inhibition and scavenger receptor null mice inhibited phagocytosis.

WS1.26

Effects of Dietary Gluten on Murine Natural Killer Cell Activity

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Dietary gluten influences the development of type 1 diabetes (T1D) in non-obese diabetic (NOD) mice and biobreeding (BB) rats and has been shown to influence a wide range of immunological factors in the pancreas and gut. A recently published case report describes a boy with T1D, who was able to maintain a low fasting blood glucose level without insulin therapy for more than 20 months, while adhering to a gluten-free diet since the time of diagnosis. In this study, the effect of gluten on NK cells *in vitro* and *in vivo* was studied.

In vitro gliadin increased direct cytotoxicity and IFN-γ secretion from C57/BL6 splenocytes against MIN6 cells, and gliadin preincubation of MIN6 cells led to a significantly increased proportion of degranulating C57/BL6 $CD107a^+$ NK cells. Stimulation of C57/BL6-purified NK cells with gliadin increased secretion of TNF-α, IL-2 and IL-10 after only 2 h of preincubation with MIN6 cells.

In vivo the standard gluten-containing diet led to an increase in $NKp46^+$ cells in BALB/c pancreatic lymph nodes (PLN) compared to a GF diet. Finally, we found higher expression of the proliferation marker CD71 and activating receptor NKG2D on $NKp46^+$ cells in all lymphoid organs in BALB/c and NOD mice receiving the gluten-containing diet.

We suggest that gluten peptides may activate NK cells locally in the pancreas or gut, which may be important during onset of T1D. These results represent a possible mechanism for the effect of gluten on T1D and may help to understand the beneficial effects of a GF diet seen in human disease and animal models of T1D.

WS1.27

Cholera Toxin Selectively Blocks TLR7 Expression in Human Lymphocytes

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Cholera toxin (CT) is an enterotoxin from *Vibrio cholera*, the causative agent of cholera disease in humans. Toll-like receptors (TLR) are a family of pathogen-recognition receptors sensing a variety of pathogens, critical for the effective innate immune response to infection. The regulation of TLR expression depends on a variety of stimuli such as microbial components and cytokines. In this study, we investigated whether CT could modulate TLR expression and thus interfere with the TLR-mediated response. We found that CT specifically inhibited TLR7 mRNA expression in peripheral blood mononuclear cells (PBMC) as well as in TLR7-expressing plasmacytoid dendritic cells and B cells. The TLR7 downregulation was mediated through the catalytic CTA portion but was independent of cAMP induction and consequent PKA activation. Furthermore, although a PKC activator mimicked the results obtained by CT, the PKC inhibitors could not reverse the CT-mediated downregulation of TLR7. However, we found that CT induced the secretion of IL-6 and IL-8 and these cytokines promoted the inhibition of TLR7 expression in PBMC. This implies that CT induces downregulation of TLR7 via an autocrine induction of inflammatory cytokines.

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Workshop 2. Immune Cells, Development and Differentiation/Lymphocyte Activation and Function

WS2.1

Differential Expression of TIM-2 During B Cell Development

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Transmembrane (or T cell) immunoglobulin and mucin domain containing molecule 2 (TIM-2) is expressed by mature T_{H2} and B cells in mouse. Interestingly, our recent report has shown that TIM-family molecules are also expressed during embryonic haematopoiesis in mouse and chicken; TIM-1 is expressed in embryonic para-aortic/aorta-gonads-mesonephros region and TIM-4 by foetal liver myeloid progenitor cells. Here, we investigated the role of TIM-2 in haematopoiesis. Our results show that TIM-2 is expressed in foetal liver and adult bone marrow by common lymphoid progenitor, but not by myeloid progenitor cells. Furthermore, foetal liver $CD45^+TIM\text{-}2^+$ cells have *in vitro* B lymphoid but not myeloid potential. During B cell differentiation, large pro-B and large pre-B cells express TIM-2 in both foetal liver and adult bone marrow. However, the subsequent small pre-B and immature B cells do not express TIM-2 until differentiation into transitional B cells. Because earlier studies have reported TIM-2 to function in the inhibition of cell proliferation, the possible role of TIM-2 during B cell proliferation is discussed.

WS2.2

Prospective Isolation of Committed Mast Cell Progenitors from Mouse Blood

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Introduction: Mast cell progenitors develop from haematopoietic stem cells in the bone marrow. Progenitors committed to the mast cell lineage are postulated to circulate through the blood and mature when reaching the peripheral organs. However, committed mast cell progenitors in the blood have not been found in mice or humans.

Methods: Prospective mast cell progenitor populations from mouse blood were isolated using gradient centrifugation and two rounds of fluorescent-activated cell sorts. The sorted cells were cultured in a cytokine cocktail, which allowed differentiation into all myeloerythroid lineages.

Results: We isolated a population of $lineage^-$ $c\text{-}kit^{hi}$ $IL\text{-}33R^+$ integrin $\beta7^{hi}$ $CD16/32^{hi}$ progenitors from blood, constituting 0.0045% of the mononuclear cells, which gave rise to a pure population of mature mast cells when cultured. The blood mast cell progenitors expressed higher levels of FcεRI in Th2-prone BALB/c mice than in Th1-prone C57BL/6 mice.

Conclusions: We have identified a population of committed mast cell progenitors in mouse blood. These cells were more mature in Th2-prone than in Th1-prone mice based on the expression of FcεRI.

WS2.3

Trojan, a Possible Regulator of Apoptosis, Belongs to a Novel Protein Family

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Trojan is a novel transmembrane protein, cloned from chicken embryonic cDNA library that belongs to a yet uncharacterized family of proteins. The molecule is predicted to have an extracellular CCP domain, followed by two FN3 domains and a short cytoplasmic tail with several overlapping short functional motifs.

Trojan is expressed specifically in lymphoid tissues, where its expression pattern is similar to that of IL-7Rα and the antiapoptotic Bcl-2 on developing thymocytes. *In vitro* studies showed that upon apoptosis induction, Trojan expression rises on the surface of surviving cells and gradually decreases towards its normal levels as cells recover and proliferate. This similar expression, supported by our current functional analyses, makes Trojan an attractive candidate of having an antiapoptotic or proliferative role. Stimulation through Trojan has yielded controversial results that tend to show a slight rise in the intracellular Ca^{++}. We are aiming to mimic cell–cell and ligand binding interactions in order to acquire conclusive results.

The family of Trojan includes two other members. One is a receptor type protein tyrosine phosphatase and the other is a membrane protein having an ITAM within its cytoplasmic tail. By doing phylogenetic analyses, we determined that the three family members had been subject to positive evolutionary selection.

In the present study, we characterize a novel avian leucocyte protein and its family evolution. From the data acquired, we expect Trojan to serve as an apoptosis regulator.

WS2.4

Improving the Quality of Dendritic Cells to be Used in Immunotherapy

Dag Heiro Yi & Silke Appel

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Therapeutic vaccinations of patients with malignant diseases are based on the stimulation of their own immune systems in order to get an antitumour response. Therapeutic vaccines using dendritic cells (DC) have been shown to be well tolerated and safe. However, this method requires an improvement, as only few clinical relevant responses could be documented so far. OK432 (picibanil) is an immunotherapeutic agent derived from penicillin-killed Streptococcus pyogenes that has been used effectively to treat a variety of tumours. It has been shown previously that OK432-stimulated DC produce large amounts of IL-12. We here investigated the possibility to reduce the time used to generate monocyte-derived DC (moDC) using OK432 in combination with TLR ligands and PGE2 to stimulate the cells. 6-day DC cultures and 3-day DC stimulated with a cytokine cocktail consisting of IL-1beta, TNF-alpha, IL-6 and PGE2 were used as controls. The combination of OK432, TLR ligands and PGE2 used to stimulate moDC after 3 days of culture resulted in phenotypically mature cells expressing high amounts of co-stimulatory molecules, MHC class II and CCR7. These cells performed at least equally well in an MLR as 3-day moDC cultures stimulated with the cytokine cocktail. Moreover, preliminary data suggest that these cells produced considerable amounts of IL-12. These results make 3-day moDC stimulated with a combination of OK432, TLR agonists and PGE2 a promising alternative to be used for moDC-based cancer vaccines.

WS2.5

Structural Biology Reveals Binding Modes of Superantigen SEE to TCR

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Superantigens (SAgs) are bacterial toxins that are able to cause a massive immune response, which may lead to food poisoning and toxic shock syndrome. *Staphylococcus aureus* is a bacterium that can produce a broad range of SAgs, that is, staphylococcal enterotoxins. A deeper insight into how bacteria act on humans is necessary, because bacteria are constantly developing resistance against antibiotics, whereas not as many novel antibiotics are discovered. SAgs are capable of cross-linking T cell receptors (TCRs) and major histocompatibility complex (MHC) class II. They bind to TCR variable α domain (Vα) or β domain (Vβ). Most SAgs utilize the TCR Vβ, although there are differences in binding specificity. The staphylococcal SAgs are classified into five different taxonomic groups (I-V), based on their protein sequences. SAg-TCR structures with SAgs from group I, II, IV and V are available. We have determined the X-ray structure of staphylococcal enterotoxin E (SEE), from group III, in complex with its TCR to 2.5 Å resolution. The TCR-SAg structure shows how SEE binds specifically to Vβ of the TCR, via extended loops. Many hydrogen bonds are observed between the SEE and TCR. Structural knowledge of SAgs in complex with TCR helps in understanding how SAgs cause massive immune responses.

WS2.6

Lipid Subdomains and the Actin Cytoskeleton in B Cell Receptor Organization and Signalling

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B lymphocytes are responsible for humoral immunity, one of the main components of the immune system protecting the individual from pathogens. Binding of antigen with B cell receptor (BCR) starts an intracellular signalling cascade ultimately leading to antibody production. Aberrant B cell activation plays a role in several diseases, such as immunodeficiencies, autoimmunity disorders and cancer. Recently, it was demonstrated that the compartmentalization of BCR, regulated by underlying actin cytoskeleton, controls the receptor signalling even bypassing the need of ligand binding. An outstanding and completely unresolved question is how the lipid microenvironment contributes to this behaviour of BCR and how the actin cytoskeleton and plasma membrane interact to provide optimal regulation for signalling. Advanced super-resolution optical microscopic techniques enable unprecedented observations of these nanodomains in live cells. Our aims are:

1 to determine the direct role of Lo membrane domains in mediation of B cell receptor organization, activation, dynamics and signalling.
2 to determine the interaction between the different plasma membrane domains and cortical actin cytoskeleton.

At the moment, we are setting up live cell imaging techniques including super-resolution microscopy such as single-particle tracking and dSTORM. In our study, a novel specific Lo domain binder is used. Ostreolysin (Oly) is a 16-kDa cytolytic protein specifically expressed in Pleurotus ostreatus. Oly binds only to membrane containing equimolar sphingomyelin and cholesterol in Lo phase, where cholesterol concentration is over 30% of all lipids. As such, modified non-lytic Oly is ideal marker for Lo domains

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suitable for live cell imaging. In addition, we are using laurdan two-photon microscopy and lipid domains markers based on differentially lipidated GFPs, as well as phosphoinositide markers. With the combination of mentioned techniques, we are able to study the role of different lipid compartments and plasma membrane microdomains in receptor signalling, actin cortex and cell activation.

WS2.7

Identification of Novel Regulators of B Cell Receptor Signalling

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The protection of individual from surrounding pathogens requires coordinated action of the innate and adaptive arms of the immune system. B lymphocytes play a crucial role in adaptive immune system by producing antibodies that neutralize bacteria and toxins and target infected or malignant cells for destruction. B cells recognize pathogens, or pathogen-derived antigens, by their B cell receptor (BCR), which then triggers intricate downstream signalling pathways and ultimately leads to cell differentiation and antibody production. Strikingly, almost nothing is known how the signals are transmitted in space and time in the cell to trigger subsequent and necessary physical changes, like antigen internalization and processing, and cellular morphology changes. To understand the molecular mechanisms governing B cell activation, it is essential to understand the functional significance and the interaction between BCR and various signalling pathways and to identify the major players at different stages of activation (i.e. early BCR signalling, internalization, antigen processing). To this end, we use newly established techniques, called BioID and APEX, which allow identification of novel proteins that interact with or are in the close vicinity of BCR during the different stages of cell activation. As the methods are based on biotinylation inside intact cells and are highly controllable in time, we gain unprecedented insight into the molecular mechanisms of BCR signal transmission with high spatial and temporal precision. Currently, we are validating our BioID and APEX constructs and estimate to get first results of the BCR proximity interactome in 6 months.

WS2.8

GSH and Trx Can Substitute for Each Other During dNTP Synthesis in Human $CD4^+$ T cells

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$CD4^+$ T cells play a very central role in the immune system, as they modulate the response to a particular pathogen. When the immune system is activated, the T cells recognizing the specific antigen need to be activated and expand in numbers. Expansion requires that the cells are activated, grow, replicate their DNA and divide.

We have previously shown that T cells express transporters for the amino acid cysteine when activated and that the uptake of cysteine is required for DNA synthesis. In this study, we show that cysteine is needed for glutathione (GSH) synthesis and that in the absence of cysteine in the medium, activated T cells fail to synthesize GSH. The synthesized GSH is needed for DNA synthesis, which explains why T cells depend on cysteine uptake to be able to synthesize DNA. Some residual DNA synthesis can take place in cells depleted of GSH. However, this residual DNA synthesis could be abrogated by the inhibition of thioredoxin (Trx), showing that Trx can substitute for GSH during DNA synthesis in $CD4^+$ T cells.

In other organisms, GSH and Trx have been shown to be electron donors for the enzyme RNR during synthesis of the DNA building blocks, dNTPs. The failure to reduce RNR causes DNA synthesis to stop. In this study, we show that inhibition of both Trx and GSH reduces dNTP levels compared to cells in which only one of these antioxidants is inhibited and untreated cells. These data provide evidence of GSH and Trx being needed for dNTP synthesis, most likely by acting as electron donors for RNR.

WS2.9

A Ternary Complex of the T Cell-Specific Adapter Protein (TSAd), Lck and IL-2 Inducible Kinase (Itk) is Maintained by Mutual Phosphorylation Events

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T cells play a major role in adaptive immune response, protecting the host from infections and cancer. Activation of T cells is tightly controlled by kinases, phosphatases and adapter proteins. Crucial steps in T cell activation is the

activation of the kinases Lck and Itk. The T cell-specific adaptor protein (TSAd) is expressed predominantly in activated T cells and interacts with both Lck and Itk. We have previously shown that Lck-mediated phosphorylation of Itk is promoted by TSAd. Moreover, TSAd also promotes phosphorylation of Lck-Y192, which changes the binding affinity of the Lck-SH2 domain and results in increased binding of Lck to TSAd. Here, we use a combination of siRNA-mediated suppression of TSAd in Jurkat T cells as well as transient transfections in 293T cells and Jurkat T cells, and report that TSAd promotes Lck-Itk interaction. Tyr to Phe mutants of either TSAd or Lck point to critical roles for Lck-Y192 and TSAd-Y290 in maintaining the three-molecular complex of TSAd, Itk and Lck. TSAd-Y290 is indispensable for TSAd's effect on Lck-mediated Itk phosphorylation. The emerging picture is thus that interaction of TSAd with Lck and Itk may be reinforced by reciprocal phosphorylations. The role of this ternary complex in events downstream of TCR activation is currently under investigation in our laboratory.

WS2.10

Colon Draining Lymph Nodes Act as Secondary Sites of Activation for Autoreactive BDC2.5 T Cells in NOD Mice

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The studies on lymphatic system and activation of specific immune responses are extensive; however, little effort has been put on structural studies of lymphatic vasculature. Nevertheless, this question is of paramount importance when making claims on activation of cell populations due to occurrences in specific organs. Here, we concentrate on the colon draining lymphatic system and the mesenteric lymph nodes (MLN), and how the lymph veins collect lymphatic fluids from different parts of the colon to specific LNs. To visualize lymphatics and primary LNs, we injected fluorescein isothiocyanate-labelled dextran (FITC-Dx) subserosally to various sites of colon. The ascending, transverse and descending colon each drain to individual LNs embedded in the mesenterium. The activation of plasmacytoid and $CD103^+$ dendritic cells was found to be activated at these sites after the induction of colitis, whereas the major part of MLN did not show activation of these cell populations. We also saw a significant activation of autoreactive BDC2.5 T cells in colon draining LNs (coLNs), but not in MLN. These results underline the importance of recognizing the relevant lymphatic sites according to the primary organ which is to be studied.

WS2.11

The Role of Pro-Inflammatory Cytokines upon the Differentiation and Function of In Vitro-Induced $CD8^+$ Tregs

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Introduction: $CD8^+$ Tregs may play an essential role in the immunopathology of various autoimmune diseases. Therefore, they have a great potential as therapeutic agents and further studies on their behaviour are essential to enhance our understanding regarding their function. The aim of this study was to evaluate the role of the innate immune system on *in vitro*-induced $CD8^+$ Treg differentiation and function. Furthermore, their cytokine profile was investigated.

Material and methods: Naive human $CD8^+CD25^-CD45RA^+$ T cells were selected from PBMCs isolated from healthy blood donors and cultured in Treg-inducing conditions with or without IL-1β, TNF-α or monocyte-derived DCs. Their cytokine profile was analysed using ELISA and Luminex assay.

Results: $CD8^+CD127^-CD25^{hi}FoxP3^{hi}$ Tregs ($CD8^+$ iTregs) were induced *in vitro* in the presence of TGF-β1 and IL-2, which had a synergistic effect ($P < 0.0001$) upon their differentiation. Various concentrations of IL-1β and TNF-α were added to the $CD8^+$ T cell inducing cultures. IL-1β significantly suppresses the differentiation of human $CD8^+$ iTregs at high concentration ($P < 0.01$). Furthermore, IL-1β (10 ng/ml) and TNF-α (50 ng/mL) reduced the $CD8^+$ iTreg-mediated suppression on both $CD4^+$ and $CD8^+$ effector T cells. This may be linked to the reduction in IL-10 and TGF-β1 secretion ($P < 0.05/0.01$) in the presence of IL-1β and TNF-α. Finally, the role of innate immunity in the differentiation and function of $CD8^+$ iTregs was further verified in the presence of mDCs. In addition, their function was possibly associated with TGF-β1 secretion.

Conclusion: The generation of functional $CD8^+$ iTregs is IL-2 and TGF-β1 dependent under both anti-CD3- and mDC-driven stimulatory conditions. In addition, TNF-α and IL-1β reduce their suppressive function, which might be IL-10 and TGF-β1 driven. Thus, this study provides clear evidence that cells and molecules of the innate immune system have a great influence upon the differentiation and function of human $CD8^+$ iTregs.

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WS2.12

Differences in the Autoreactive T Cell Pool When Restricted by the Highly Related Rheumatoid Arthritis-Associated HLA-DRB1 Alleles *04:01, *04:04 and *01:01

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The shared epitope (SE) alleles HLA-DRB1*04:01, *04:04 and *01:01 are associated with risk for rheumatoid arthritis (RA) positive for autoantibodies against citrullinated proteins, for example α-enolase. Recent studies identified specific citrullinated epitopes presented by *0401 that are associated with disease and provided molecular evidence for how citrullination could break tolerance. So far, the risk conferred by *04:04 and *01:01 is less well characterized. Here, we aimed at the identification and functional characterization of citrullinated α-enolase T cell epitopes presented by these alleles.

Overlapping peptides were screened and several novel α-enolase epitope candidates identified. A large, but not complete, overlap was observed between *0401, *0404 and *0101. Surprisingly, the binding of native and citrullinated epitopes indicated that the bias for citrullinated epitopes of *04:01 appears less pronounced in *0404 or *0101. *In vitro* stimulation of peripheral blood mononuclear cells (PBMCs) demonstrated that a number of the epitope candidates are indeed immunogenic in RA patients and elicit a pro-inflammatory cytokine response. However, and in agreement with the peptide binding, a significant preference for citrullinated epitopes was only observed for *04:01.

While this study confirms the compelling bias of *04:01 for citrullinated epitopes, it demonstrates its absence in *0404 and *0101. Our data suggest that, despite their name and shared amino acids, significant differences exist in how these HLA-DR alleles confer risk to RA. Altogether, this demonstrates how even subtle changes in the MHC peptide-binding groove can have significant immunological impacts and has prompted ongoing structural characterization of the different properties of *04:01 versus *01:01 and *04:04.

WS2.13

Retinoic Acid Modulates the Early Expansion and Differentiation of $CD4^+$ T Cells During the Development of Intestinal Inflammation

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Epidemiological studies of vitamin A-deficient populations have illustrated the importance of vitamin A and its metabolite all-trans retinoic acid (RA) in mucosal immune responses. In support of that finding, we and others have shown that RA produced by intestinal dendritic cells is required for the generation of gut-tropic T cells and IgA-secreting plasma cells. Evidences from the literature suggest antagonistic roles for RA in intestinal T cell biology. In the homeostatic intestine, RA plays anti-inflammatory functions, sustaining the development of $foxp3^+$ regulatory T cells, while restraining Th17 effector T cell differentiation. In contrast, RA has pro-inflammatory functions, promoting Th1/Th17 effector T cell responses, in the gut upon pathogen exposure. Therefore, the circumstances in which RA plays pro- or anti-inflammatory roles and the target T cell populations remain to be more clearly defined.

RA has been shown to control the $CD4^+$ T cell compartment by signalling through the RA receptor RARα. We therefore took advantage of mice expressing a dominant negative form of the RARα, specifically in the CD4 T cell compartment ($RAR^{fl/wt}$ $CD4Cre^+$ mice), in order to conditionally ablate RA signalling in $CD4^+$ T cells during the development of intestinal inflammation in the T cell transfer colitis model.

Histological, flow cytometry and real-time PCR analyses indicate that RA signalling modulates the early expansion and differentiation of $CD4^+$ T cells following their transfer into $RAG^{-/-}$ mice. Indeed, RA signalling-deficient $CD4^+$ T cells selectively accumulate and showed capacities to differentiate into IL-17^+, IL-17^+IFN-γ^+ and $foxp3^+$ cells, but reduced capacities to differentiate into IFN-γ^+ cells and were less potent at inducing intestinal inflammation. RA signalling-deficient $CD4^+$ T cells were also unable to differentiate into cytotoxic T lymphocyte (CTL)-like cells within the intraepithelial lymphocyte compartment of the colon.

We are currently deciphering the mechanisms of these effects of RA on T cells.

WS2.14

Abolishing Riboflavinkinase Activity Massively Impairs a T Cell-Mediated Antiviral Immune Response

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Riboflavinkinase (RFK) activity generates FMN, which is also the substrate for synthesis of FAD. Both FMN and FAD are essentially required by flavoenzymes in a multitude of metabolic pathways. The consequences of RFK deficiency during the immune response against the lymphocytic choriomeningitis virus (LCMV) were characterized in conditionally (RFK(fl/fl)/MxCre) or cell specifically (RFK(fl/fl)/CD11cCre or CD4Cre) RFK-deficient mice.

Systemic levels of type I IFN early after infection of mice with LCMV effectively induced Cre expression in RFK(fl/fl)/MxCre mice as judged by rapid deletion of floxed RFK alleles and, consequently, complete knockdown of RFK at the protein level by day 4 post-infection. As a consequence, proliferation and maturation of LCMV-specific $CD8^+$ T cells were massively impaired. This resulted in significantly delayed elimination of LCMV from the organs of RFK(fl/fl)/MxCre mice.

To reveal in which cell type(s) RFK activity is required to generate antiviral $CD8^+$ T cells, the LCMV-specific immune response was characterized in mice with cell type-specific deletions of RFK either in dendritic cells (RFK(fl/fl)/CD11cCre) or in T lymphocytes (RFK(fl/fl)/CD4Cre). In RFK(fl/fl)/CD11c Cre mice, the generation and antiviral activity of LCMV-specific $CD8^+$ T cells were not significantly impaired. In sharp contrast, in RFK(fl/fl)/CD4Cre mice, the expansion and maturation of LCMV-specific $CD8^+$ T cells were almost abolished. Consequently, the LCMV was not eliminated from the organs of these mice.

Taken together, deletion of RFK activity even during an ongoing virus infection significantly suppresses the virus-specific immune response. Therefore, inhibition of RFK activity might be a promising approach for immunosuppressive therapy in future.

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Workshop 3. Immune Regulation

WS3.1

Divergent Regulation of B and T Cell Responses by Complement-Activating IgM

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Complement is important for immune responses and IgM is a potent activator of the complement system. However, knockin mice of the Cμ13 strain, producing IgM unable to activate complement due to a point mutation on the μ-heavy chain, have normal primary antibody responses. This suggests that natural IgM capable of activating complement does not play a key role in inducing normal antibody response. This puzzling finding promoted our investigation of whether the ability of IgM to activate complement is indeed required in specific IgM-mediated enhancement of immune responses. We first compared the binding ability of the mutant Cμ13 IgM and WT IgM to the recently discovered murine IgM Fc receptor (FcμR) and demonstrated that both of them bind equally well to the FcμR. In spite of this, WT IgM enhanced antibody and germinal centre responses after immunization together with sheep red blood cells (SRBC) or keyhole limpet hemocyanine (KLH), whereas Cμ13 IgM failed to do so. In addition, it has been reported that IgM cannot enhance antibody responses in T cell-deficient nude mice and thus does not substitute for T cell help. Interestingly, we found that WT IgM anti-SRBC administered with SRBC-OVA had no effect on the activation or proliferation of OVA-specific $CD4^+$ T cells although it efficiently enhanced the T-dependent antibody responses against both SRBC and OVA. In conclusion, our study emphasizes the importance of complement-activating ability of specific IgM to enhance B cell responses and also reveals a divergent regulation of T and B cell responses by IgM.

WS3.2

Antigen-Specific IgM Causes Deposition of C3 on Sheep Red Blood Cells Within Seconds After Immunization

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C1q, the initiator of the classical pathway, is primarily activated by antibody–antigen complexes (immune complexes, ICs). Deficiency of classical complement factors results in impaired antibody responses. This may seem as a paradox, because there are no, or are least very low, antigen-specific antibodies (forming the ICs that activate C1q) prior to the antibody response. Nonetheless, if antigen-specific IgM is administered before the antigen, the antibody response will be enhanced: IgM-mediated enhancement – a phenomenon dependent on IgM being able to activate complement. It was thus hypothesized that natural IgM would bind an antigen with enough affinity to form the ICs C1q needed for activation. However, this was proven wrong when it was shown that Cμ13 mice (producing IgM unable to activate complement) have normal antibody responses. This prompted the investigation of whether the antigen itself, in this case sheep red blood cells (SRBC), was able to activate complement. C3 deposition on SRBCs incubated 30 min with fresh mouse plasma did not differ from SRBC incubated with PBS. Only after adding IgM anti-SRBC to the mixture, there was a marked C3 deposition on the SRBC. *In vivo*, immunization with SRBC together with specific IgM resulted in C3 deposition on the antigen within seconds in the blood. SRBC administered alone had no detectable C3 on their surface. Thirty minutes after immunization with SRBC alone, C3-coated SRBC were found in the marginal zone. In conclusion, SRBC alone are able to activate complement *in vivo*, although specific IgM markedly increases the efficiency of C3 deposition.

WS3.3

MZ B Cells Transport IgG3-Ag to Splenic Follicles and Induce Germinal Centres

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IgG3–antigen complexes can cause enhancement of the antigen-specific antibody response compared to what is seen when the antigens are administered alone. According to our *in vivo* study, IgG3 increases antigen binding to marginal zone (MZ) B cells, but not to peripheral B cells. The binding is mediated through complement receptors 1 and 2 (CR1/2, encoded by *Cr2*). Within 2 h of immuni-

zation, antigen complexed to IgG3 can be detected in splenic B cell follicles. This is followed by an increased germinal centre formation compared to that seen in mice immunized with antigens alone. Experiments with Cr2 KO mice show that the IgG3-mediated effects depend on CR1/2. Studies in bone marrow chimeric mice demonstrate that CR1/2 must be expressed on both follicular dendritic cells (FDC) and B cells in order for an optimal IgG3-mediated enhancement of antibody responses. Thus, these data suggest that CR1/2^{+} MZ B cells transport IgG3–antigen immune complexes to B cell follicles, thereby increasing the concentration of antigen as well as the germinal centre formation, which leads to the enhancement of antibody responses.

WS3.4

Anti-CD23–Antigen Conjugates Localize Antigen to Splenic Follicles and Enhance Specific Ab Response

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Antigen transported to lymphoid B cell follicles is the initial step of adaptive immune responses. Follicular B cells (FOBs) circulate from peripheral blood through the secondary lymphoid organs. Therefore, they are ideal transporters of antigens as they have access to splenic follicles. Here, we report that targeting antigens to CD23, a receptor on FOBs, is followed by rapid transport of the antigens to follicles. Anti-CD23 mAb (or an isotype control) were covalently linked to OVA and administered *i.v.* to mice. The antigen binds to FOBs in peripheral blood 10 min after immunization and is localized in splenic follicles after 30 min. Specific Ab responses were also enhanced in mice immunized with anti-CD23-antigen as compared to isotype control antigen or antigen alone. Moreover, anti-CD23–antigen conjugates did not enhance specific antibody responses or localization of antigen to splenic follicles in CD23 KO mice, demonstrating that these processes are CD23 dependent. Our data suggest a possible way to enhance specific antibody responses and may be useful for therapeutic vaccination.

WS3.5

IgG-Mediated Suppression of Primary IgG Anti-SRBC Responses is not Dependent on FcγR or Complement

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Antibodies administered with the antigen to which they bind can profoundly influence the resulting antigen-specific humoral immune response, a phenomenon known as antibody feedback regulation. This regulatory capacity can result in 99% suppression or 1000-fold enhancement of the specific antibody response and is dependent on antigen and antibody isotype. Passively administered IgG can suppress the specific antibody response against large particulate antigens such as erythrocytes. This is used to prevent haemolytic disease in newborns by so-called Rh(D) prophylaxis. The mechanism behind IgG-mediated suppression is not very well understood. One possibility would be that IgG could interact with the inhibitory Fc-gamma receptor (FcγR) on B cells (FcγRIIB) and/or other FcγR and exert its suppressive function via these. Alternatively, the complement system could be involved. Earlier studies have shown that suppression of the IgM response does not require FcγR. The aim of the present study was to investigate whether IgG could suppress IgG responses, whether this required FcγR, and whether IgG could suppress in mice lacking CR1 and CR2 or C1q.
Mice (BALB/c, FcRγ$^{-/-}$, FcγRIIB$^{-/-}$, Cr2$^{-/-}$, C1q$^{-/-}$) were immunized i.v. with IgG anti-SRBC (sheep red blood cells) and SRBC or with SRBC or IgG alone. IgM (direct plaque-forming cells, PFC) and IgG (ELISA) responses were analysed and, as expected, IgG suppressed these responses dramatically. The suppressive capacity of IgG was not affected by the absence of FcRγ, FcγRIIB, CR1/CR2 or C1q.

WS3.6

Studies on the Human Primary Amine Oxidase/Sialic Acid Binding Ig-Like Lectin-9 Interaction

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Leucocyte trafficking from blood vessels into inflamed tissues is essential in maintaining health. Human primary amine

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oxidase (hAOC3) on the endothelium is a significant player in the trafficking with a still unknown mechanism. The first hAOC3 counter molecules have been identified being sialic acid binding Ig-like lectins-9 and -10 [SIGLEC-9 and -10; Kivi *et al.* (2009) *Blood* 114:5385; Aalto *et al*. (2011) *Blood* 118: 3725]. Our objective is to characterize the mechanisms of hAOC3 in the leucocyte trafficking by studying its interaction with SIGLEC-9 and -10.

For the interaction studies, we have put up an insect cell production system and purified the protein by chromatographic methods. The binding constants for hAOC3/SIGLEC interaction were determined by a surface plasmon resonance (SPR). The mutagenesis of specific arginine residues of SIGLECs was performed to study the importance of these residues in the interaction. We also studied the activity of hAOC3 towards the recombinant SIGLECs. We will aim for detailed characterization of the interaction via the co-crystallization of hAOC3/SIGLEC complex. Finally, we will test whether hAOC3/SIGLEC interaction influences SIGLEC signalling in neutrophils.

We have been able to produce a soluble extracellular part of SIGLEC-9. The SPR studies indicate specific binding of SIGLECs to the immobilized hAOC3, with Kd of about 1 micromolar. The arginine residues, which were shown by peptide studies to be important for the binding, do not affect significantly the binding at the protein level. However, we have demonstrated that the binding of SIGLEC-9 to hAOC3 is dramatically affected by the addition of sialic acid to the binding assay. This implies that also sialic acid-binding V-domain is involved in the SIGLEC-9/hAOC3 interaction. In addition, we have demonstrated that SIGLEC-9 may interfere the activity of hACO3.

WS3.7

Modulation of Dendritic Cells by Exosomes Derived from Breast Milk and Plasma

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Exosomes are small extracellular vesicles, 30–100 nm in diameter, produced and secreted by most cells including immune cells. They play a role in a variety of biological systems and are capable of both immune activation and tolerance induction. Breast feeding has been suggested to decrease the risk of allergy development in the newborn child. Breast milk contains exosomes that can induce regulatory T cells in a mixed peripheral blood mononuclear cell culture. However, it is not known whether milk exosomes exert a direct effect shifting naïve T cells to a regulatory phenotype or whether exosomes induce a tolerogenic dendritic cell phenotype that in turn regulates the T cell profile. In contrast to milk exosomes, we speculate that plasma exosomes are more pro-inflammatory. Therefore, we compared these two exosomal sources to explore whether they have different immune modulatory properties. Exosomes were isolated by differential ultracentrifugation and captured on anti-CD63-coated latex beads for phenotype analysis using flow cytometry. Monocyte-derived dendritic cells (MDDCs) were incubated for 24 h with exosomes from milk or plasma, respectively, followed by phenotyping using flow cytometry. Milk and plasma exosomes expressed similar surface markers such as the tetraspanins, CD9, CD63 and CD81 but had low HLA-ABC and HLA-DR levels. In spite similar levels of surface molecules, plasma-derived exosomes, but not milk derived, strongly increased HLA-DR expression on MDDCs. These data indicate that exosomes of different origin carry diverse biological functions. While plasma exosomes seem to be more immune stimulatory, we are currently investigating the tolerogenic capacity of the milk exosomes.

WS3.8

Requirement for IRF4-Dependent DCs for the Generation of Th2 Responses During Infection with the Murine Gastrointestinal Nematode Trichuris muris

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The nature of adaptive $CD4^+$ T cell responses is controlled by the antigen-presenting DC and environmental factors present during activation. While the requirements for the induction of Th1, Th17 and regulatory T cells are relatively well defined, comparably little is known with regard to how $CD4^+$T cells are directed to become Th2 cells, including the involvement of specific DC subsets. *Trichuris muris* is a large intestinal nematode frequently used to study Th2 responses. Here, we investigate the role of DC subsets during *T. muris* infection, utilizing wild-type mice and CD11cCRE × *IRF4*flox mice that have specific reduction in $CD103^+CD11b^+$ and $CD103^-CD11b^+$ DCs in the mesenteric lymph nodes (MLN) and intestine. Preliminary results show an aberrant Th2 cytokine profile in MLNs and increased worm burden in infected mice with IRF4-deficient DCs, suggesting an impaired Th2 response in these mice. This suggests that *IRF4*-dependent DCs are important for the generation of optimal Th2 responses during intestinal helminth infection. We are currently utilizing *in vitro* models to further investigate DC subsets and mechanisms involved during Th2 induction.

WS3.9

Interferon Regulatory Factor 4 Expression in Thymic Epithelium Regulates Peripheral Tolerance by Controlling Thymic Regulatory T Cell Output

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Interferon regulatory factor 4 (Irf4) has been shown to play a critical role in the differentiation and function of a diverse group of peripheral immune cell populations including B cells, macrophages, dendritic cells and several T cell subpopulations. Despite its high expression levels in thymic epithelial cells (TECs), Irf4 has not been studied in the context of thymic microenvironment and its consequent effect on the generation of an immunocompetent set of peripheral T cells. To address this issue, we crossed *Irf4 flox/flox* mice with *FoxN1-Cre* mice eventually resulting in TEC-specific Irf4-deficient progeny ($Irf4^{-/-}$). Here, we show that the morphology of Irf4-deficient thymi is in general unaffected and the numbers and phenotype of a majority of T cell populations originating from the thymus appear to be comparable to the control mice. We witnessed, however, a 50% decrease in the numbers and percentages in regulatory T (T_{reg}) cells in the thymi of 2-month-old $Irf4^{-/-}$ mice. This decrease was also present in the periphery where $Irf4^{-/-}$ mice had a 20% decrease in the splenic T_{reg} cell population. Aged $Irf4^{-/-}$ mice also showed an increased incidence of autoimmune infiltrations in the salivary glands compared to the controls. Altogether, these results indicate a role for TEC-specific Irf4 expression in avoiding peripheral autoimmunity by regulating the maturation process of thymic regulatory T cells.

WS3.10

CD1d Expression on Pancreatic β-Cells as a Target for the β-Cell-Specific Autoantibody IC2

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Natural killer T (NKT) cells play a regulatory role in the immune system and are stimulated by binding to glycolipid–CD1d complexes. The autoantibody IC2 seems to have a strong inhibitory function on the cytokine release from NKT cells *in vitro*. Therefore, we searched for CD1d expression on pancreatic tumour β-cell line (β-TC-tet) and on primary mouse β-cells in order to clarify the presence of CD1d β-cell expression.

Flow cytometry was performed on spleen and pancreatic cells from NOD, NOD $CD1d^{-}$ and C57Bl/6 CD1d-knockout mice. Staining for β-cell, $CD45^{+}$, $CD1d^{+}$ and $IC2^{+}$ cells was performed. For confocal microscopy studies, the $IC2^{+}$, $CD1d^{+}$ and insulin were used. The staining was performed according to the standard protocols. Classical iNKT functional assays with IL-2 release were used to elucidate IC2 inhibition. The hybridoma cell line NKT-1.2 functions as responder cell with CD1d-coated plates uploaded with αGalCer.

Flow cytometric analysis of both β-TC-tet and primary β-cells from the three mice types demonstrated CD1d on their surface. Expression of CD1d on β-cells was observed to be significantly higher, compared to the expression on $CD45^{+}$ cells from pancreas and spleen. Furthermore, IC2 binding is shown on β-TC-tet and primary β-cells both in flow cytometry and in confocal microscopy. Flow cytometric data indicate CD1d expression on β-cells from the two CD1d-knockout mice strains.

These observations indicate that CD1d molecules are expressed not only on antigen-presenting cells but also strongly by pancreatic β-cells. The β-cell themselves can therefore contribute to the maintenance of normal NKT cells regulation. The inhibitory IC2-binding to CD1d is dependent on certain glycolipids in the cleft of CD1d, and if these lipids are not present, IC2 will not bind. We are currently searching for a CD1d glycolipid specific for β-cell and IC2 binding.

WS3.11

T Regulatory Cells Ameliorate Joint Destruction in Staphylococcus aureus-Induced Arthritis

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Background: *Staphylococcus aureus* is a common cause of bacterial arthritis, characterized by a severe destruction of the affected joints, often followed by permanent articular damage. It has proven difficult to effectively treat this type of infection due to the necessity of a maintaining a strong immune response for bacterial elimination, and concomi-

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tant downregulation of the exaggerated immune response that contributes to the tissue destruction. Regulatory T cells (Tregs), defined by the expression of CD4, CD25 and FOXP3, limit immune responses and have also been shown to inhibit the formation of bone-resorbing osteoclasts. Interleukin 2 (IL-2) is essential for the homeostasis and maintenance of Tregs in the periphery and has been shown to be of therapeutic value in treating hepatitis C-induced vasculitis by increasing and modulating Tregs. This study was performed to assess the role of Tregs in the outcome of *S. aureus*-induced arthritis.

Methods: Adeno-associated virus (AAV) vector, encoding for IL-2 or luciferase, was injected intraperitoneally into C57Bl/6 mice 19 days prior to intravenous inoculation of TSST-1-producing *S. aureus*.

Results: The IL-2-treated mice showed a profound increase in the frequency of $CD4^+CD25^+FOXP3^+$ cells (Tregs) in the blood and spleen both before and 10 days after bacterial inoculation. The IL-2-treated group lost significantly less weight and displayed an increased ability to clear the bacteria compared to the control group. Moreover, the IL-2-treated mice developed significantly decreased joint destruction and synovitis compared to the control group. These findings coincided with a significant decrease in serum levels of IL-6 in the IL-2 treated group. In addition, similar results were obtained after adoptive transfer of Tregs.

Conclusions: An increased proportion of Tregs limits the *S. aureus*-induced immune response without hampering bacterial clearance and general disease.

WS3.12

The Role of CD73 in Macrophage Polarization

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The ectoenzyme CD73 is a potent producer of extracellular adenosine as it dephosphorylates adenosine monophosphate (AMP). While CD73 can exert enzymatic-independent effects such as promoting angiogenesis, many of its functions are related to this enzymatic activity. This activity cannot only play a role in lymphocyte migration, but it can also be involved in downregulation of antitumour immunity, as the produced adenosine is highly anti-inflammatory. Due to the influence of CD73 on inflammation, its overexpression in several cancer types and its effect on cell trafficking, CD73 is an important player in the immune system.

While research on several aspects of the immune system concerning CD73 has been carried out, macrophages have so far not been studied very intensively. Macrophages can be separated into a pro- and an anti-inflammatory phenotype, also called M1 and M2. As both CD73 and macrophages are involved in (anti)-inflammatory processes, our aim was to find and characterize a connection between them. We therefore were focusing to determine whether CD73 is differently expressed on M1 and M2 macrophages and to analyse whether CD73 is required for macrophage polarization towards either phenotype.

In order to answer these questions, human macrophages were cultured and polarized towards M1 or M2 *in vitro*. In addition, mouse macrophages were polarized towards the same phenotype *in vivo*. After a successful polarization, the macrophage populations were compared by applying different techniques such as flow cytometry, qPCR and multiplex. We could clearly detect the expression of CD73 on *in vitro* M1 polarized human monocytes/macrophages, while no expression on unpolarized or M2 macrophages could be found. Furthermore, we could neither detect any difference in the CD73 expression of polarized mouse macrophages nor could we see any effect of CD73 on the polarization ability of those cells.

WS3.13

Vagus Nerve Stimulation Decreases T Cell Activation in Immune Organs

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Vagus nerve stimulation (VNS) is known to decrease the effects of an inflammatory response prompting investigations of its potential therapeutic role in chronic inflammatory conditions. The anti-inflammatory effect of VNS is mediated via interactions between neurons and T cells, as well as TNF-α-producing macrophages mainly in the spleen. However, it is still largely unknown how VNS affects the different immunologically active cell populations. Here, we aim to investigate the effect of VNS on different pro- and anti-inflammatory cell populations of the immune system, providing insight into how the anti-inflammatory properties of VNS are orchestrated.

VNS (1 V at 1 Hz for 5 min) or sham operation was performed on anesthetized male C57BL/6 mice injected intraperitoneally with 2 mg/kg LPS, respectively. After 6 h, mice were sacrificed and cells from whole blood, spleen and mesenteric lymph nodes (MLN) were acquired and stained for markers representing key lymphocytic and myeloid cell populations and their activation status.

Multicolour flow cytometry analysis revealed no differences between VNS and sham-treated mice concerning percentages of general $CD8^+$ and $CD4^+$ T cell populations as well as neutrophil and macrophage populations in the studied organs, suggesting that these populations remain

unchanged in their respective compartments after VNS. Interestingly, in MLN, we detected a significant decrease in activated $CD69^+$ $CD4^+$ T cell population in VNS-treated mice compared to sham ($3.44 \pm 0.09\%$ VNS versus $5.30 \pm 0.39\%$ sham; $P < 0.05$). Additionally, these results were in line with a decrease in activated $CD69^+$ IFN-γ^+ Th1 cells in MLN after VNS ($13.55 \pm 0.10\%$ VNS versus $27.11 \pm 6.83\%$ sham; non-significant). Similar tendencies were noted in both spleen and blood, respectively. Taken together, these data suggest that VNS may have suppressive effects on T cell activation in immune organs without changes in T cell trafficking; moreover, MLN seem to be an important site for neuroimmune interaction.

WS3.14

The Kinetics of Thymic-Derived and Peripherally Induced Regulatory T Cell Response in Early Development of Type 1 Diabetes in a Murine Experimental Model

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Background: Type 1 diabetes (T1D) has been accepted as an autoimmune disease leading to destruction of the β-cells. Despite extensive research in the field of T1D, the kinetics of the regulatory T cell (Tregs) response in early development of T1D is not yet clear. Tregs are characterized by the expression of CD4, CD25 and Foxp3 in naïve conditions. Furthermore, Helios can be used as a marker for distinguishing thymic-derived Tregs (tTregs) from peripherally induced Tregs (pTregs).

Aim: The aim of this study was to elucidate the dynamics of Tregs in the early development of T1D in the none obese diabetic (NOD) murine model.

Methods: In this study, we have examined the proportion of the Tregs subsets; $CD4^+CD25^+Foxp3^+$ (Tregs), $CD4^+CD25^+Foxp3^+Helios^+$ (tTregs) and $CD4^+CD25^+Foxp3^+Helios^-$ (pTregs) in pancreatic draining lymph nodes (PDLNs) of 8-, 13- and 20- to 21-week-old NOD female and male mice by flow cytometry. Pancreatic tissue of mice was also investigated for insulitis. The results obtained from NOD mice were compared to wild-type (wt) CD-1 mice.

Results: The proportion of Tregs was increased in both female and male NOD mice compared to wt mice. Interestingly, the proportion of IFN-γ^+ cells was increased in Tregs, tTregs and pTregs in both female and male NOD mice compared to wt mice. The degree of insulitis was higher in female compared to male NOD mice on weeks 13 and 20–21.

Conclusion: Thus, we conclude that Tregs are increased in early development of T1D. However, the upregulation does not protect from the autoimmune diabetes, possibly because of a phenotypic shift in the tTregs and pTregs population in the NOD murine model of T1D.

WS3.15

IRF4-Dependent Dendritic Cells Regulate $CD4^+$ T Cell Responses to Soluble Oral Antigens

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Dendritic cells (DCs) that migrate from the intestines to the mesenteric lymph nodes are believed to be play key roles in the initiation and regulation of adaptive immune responses to orally administered antigen; however, the *in vivo* functionality of individual DC subsets remains to be established. We and others previously showed that intestinal $CD103^+CD11b^+$ DCs are dependent on the transcription factor interferon regulatory factor (IRF)4 and are depleted in the mesenteric lymph nodes (MLNs) of mice lacking IRF4 in DCs. Using this mouse model, we here address the role of IRF4-dependent DCs in the initiation and regulation of $CD4^+$ T cell responses to soluble luminal antigen. Our results suggest that IRF4-dependent DCs are not critical for the priming of OT-II cell responses to orally administered ovalbumin (OVA). This is in contrast to our findings that IRF4 expression by DCs is required for efficient OT-II responses following intraperitoneal OVA administration. Moreover, IRF4-dependent DCs are not required for the development of induced Tregs in the MLNs or for the establishment of oral tolerance. In contrast, IRF4-dependent DCs appear to play key roles in regulating gut homing receptor expression and IL-10 production by $CD4^+$ T cells in the MLNs. In summary, our data point to an important role for IRF4-dependent DCs in the regulation of immune responses to orally administered soluble antigen.

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WS3.16

Role of Intestinal IRF8-Dependent CD103$^+$CD11b$^-$ DCs in Intestinal Immune Homeostasis

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CD103$^+$ dendritic cells (DCs) represent the major migratory DC subset in the small intestinal lamina propria (LP) and play a key role in the transport and presentation of luminal antigen to adaptive immune cells in intestinal draining mesenteric lymph nodes (MLN). CD103$^+$ DCs can be subdivided into two distinct subsets: CD103$^+$CD11b$^-$ and CD103$^+$CD11b$^+$ DC that have distinct transcription factor requirements for their development. We have recently shown that CD103$^+$CD11b$^+$ DCs are dependent on the transcription factor IRF4 for their survival and appear important in the generation of mucosal Th17 responses (Persson *et al.* Immunity 2013). In contrast, CD103$^+$CD11b$^-$ DCs are dependent on the transcription factor IRF8 (Edelson *et al.* 2010, JEM) and thus appear developmentally related to lymph node-resident CD8$^+$ DCs that are particularly efficient in cross-presenting antigen to CD8 T cells (Pooley *et al.* JI 2001, Dudziak *et al.* Science 2007). The role of intestinal CD103$^+$CD11b$^-$DCs in intestinal homeostasis remains, however, unclear. Here, we have generated mice with a specific deletion of IRF8 in their DC compartment CD11c-Cre.IRF8$^{fl/fl}$. In preliminary analysis, we have found that CD11c-Cre.IRF8$^{fl/fl}$ mice have a specific deletion of CD103$^+$CD11b$^-$ DCs within the intestinal LP and MLN. We are currently assessing the impact of loss of this DC subset has on intestinal immune compartments and intestinal adaptive immune responses.

Workshop 4. Tolerance, Autoimmunity and Transplantation

WS4.1

A Novel Connection of Autophagy-Related Gene 7 to Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

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Introduction: The autophagocytic process is linked to both innate and adaptive immunity and is implicated in neurodegeneration and autoimmunity, which are properties of multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). Genome-wide expression analysis in a backcross (BC) population of rats induced with EAE revealed a regulation of autophagy-related gene 7 (*Atg7*) expression. Therefore, we aimed to investigate the involvement of *Atg7* in EAE and MS.

Methods: Cellular source and kinetics of *Atg7* expression was studied with quantitative PCR and Western blot in sorted cells from rats. The impact of *Atg7* on the immune system and EAE development was characterized in mice with a conditional *Atg7* deletion in T cells resp. myeloid cells. *Atg7* expression was investigated in samples from MS patients and controls, and the genetic association of autophagy genes was investigated in a Swedish cohort.

Results: In rat strains, we identified *Atg7* as an EAE-associated gene and further showed that higher *Atg7* expression correlates with less severe EAE. The mouse strain with conditional *Atg7* deletion in myeloid shows aggravated EAE. In PBMC and sorted immune cells from MS patients and controls, we detected altered mRNA expression patterns. Furthermore, we observed an association of *Atg7* to MS in a Swedish population.

Conclusions: In conclusion, we demonstrate a regulation of *Atg7* expression in MS and EAE immune cells and an impact on EAE disease phenotypes. To elucidate the molecular impact of *Atg7* and autophagy in neuroinflammatory disease, further functional studies are required. Based on our findings, the primary focus is on myeloid cells targeting autophagy, regulatory and executive properties.

WS4.2

To Which Extent May the Familial Risk of RA be Explained by Established RA Risk Factors?

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Objectives: Family history of rheumatoid arthritis (RA) may be seen as an easily obtained aggregate of an individual's genetic risk and shared environmental risk factors may be seen as an easily obtained aggregate of an individual's genetic risk and shared environmental risk factors, and is accepted to be important in clinical diagnosis. It has been claimed that identified genetic factors explain 50% of RA heritability. We wished to test how much of the RA familial risk can be explained by established genetic and non-genetic risk factors.

Methods: RA disease history in first-degree relatives of individuals in the Swedish Epidemiological Investigation of rheumatoid arthritis (EIRA) case–control study was assessed through linkage to the Swedish multigeneration and patient registers. The influence of non-genetic (smoking, alcohol intake, silica exposure, BMI, fatty fish consumption, socioeconomic status) and genetic (shared epitope and 46 SNPs identified by Immunochip consortium) risk factors was assessed through logistic regression after multiple imputation.

Results: Established non-genetic risk factors did not explain any significant part of the familial risk in either anticitrullinated protein/peptide antibody (ACPA)-positive or -negative RA. Genetic risk factors explained a limited proportion of the familial risk for ACPA-positive RA ($OR_{Crude} = 4.09$, $OR_{Adjust\ for\ S.E.} = 3.72$, $OR_{Adjust\ for\ 46SNPs} = 3.44$, $OR_{Adjust\ for\ both} = 3.34$).

Conclusion: We found that established risk factors did not explain much of the familial aggregation of RA, suggesting that most heritability remains to be elucidated. In particular for ACPA-negative RA, it seems that we have only found factors responsible for a very minor (or no) part of the familial aggregation. Family history of RA remains an important risk factor for RA, and there is a need for aetiologic studies focusing specifically on ACPA-negative RA.

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WS4.3

B Regulatory Cells are Functionally Impaired in Patients with Rheumatoid Arthritis and in Their First-Degree Relatives Compared with Controls

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Introduction: B regulatory cells have been suggested to play a role in the development of various autoimmune diseases including rheumatoid arthritis (RA). In this study, the functionality of B regulatory cells in the blood from RA patients, their first-degree relatives (FDR) at risk of developing RA and healthy controls was investigated.
Methods: Mononuclear cells were isolated from 26 patients with RA, 23 FDR and 11 healthy controls from northern Sweden. Cells were analysed by flow cytometry for the expression of STAT-3, with and without anti-CD40-antibody stimulation in $CD19^{+}CD20^{+}$ $CD24^{hi}CD38^{hi}$ cells (B regulatory cells/Bregs). T regulatory ($CD4^{+}CD25^{+}FoxP3^{+}$), $CD4^{+}$ and $CD8^{+}$ cells were analysed for their expression of activation markers, such as CD69 and CD25. Seven cytokines, that is, eotaxin, interleukin (IL)-1β, IL-10, IL-12(p70), IL-6, MCP-1 and IL-17A, were analysed in plasma samples using a Luminex 200 Labmap system.
Results: When comparing the STAT-3 levels of B regulatory cells, with and without stimulation of CD40, at an individual level, a significant increase was found in controls ($P = 0.018$), while no change was found in cells from the FDR or RA patients. The cytokines IL-1β, IL-10, IL-12 (p70), IL-6, MCP-1 and IL-17A were found to be altered in RA patients as well as in FDR compared with controls.
Conclusion: The response of Bregs following stimulation was not only decreased in patients with RA but also in unaffected FDR from multicase families, with both groups differing significantly from healthy controls. This indicates that additional immunological mechanisms, and/or genetic or environmental factors, contribute to the development of rheumatoid arthritis.

WS4.4

Human Leucocyte Antigen Genes and Interferon Beta Preparations Influence Risk of Developing Neutralizing Antidrug Antibodies in Multiple Sclerosis

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Introduction: Development of antidrug antibodies (ADA) is a clinical problem associated with the use of different biological therapeutics. A significant proportion of patients with multiple sclerosis (MS) who receive therapy with interferon beta (IFN-β) develop neutralizing ADA (NAbs) that reduce drug efficacy. However, only a subset of treated patients develop NAbs, and thus, other factors are likely to influence the risk of developing NAbs. The objective of this study was to investigate whether HLA class I and II alleles are associated with the development of NAbs against IFN-β.
Methods: HLA-genotyped patients were cross-referenced to patients who had been analysed routinely for NAbs using an *in vitro* IFN-β-induced gene expression assay, resulting in a cohort of 903 patients with MS of Scandinavian origin with known HLA genotype and NAb status. Patients were treated with intramuscular IFN-β-1a, subcutaneous IFN-β-1a or subcutaneous IFN-β-1b. NAb status and development of NAb titres high enough to be biologically relevant (>150 ten-fold reduction units/ml) was correlated with HLA allele group carriage.
Results: Carriage of *HLA-DRB1*15* was associated with an increased risk of developing NAbs and high NAb titres. After stratification based on type of IFN-β preparation, *HLA-DRB1*15* carriage was shown to increase the risk of developing NAbs and high NAb titres against both subcutaneous and intramuscular IFN-β-1a. Additionally, in patients receiving subcutaneous IFN-β-1a, carriage of *HLA-DQA1*05* decreased the risk for high NAb titres. In IFN-β-1b-treated patients, *HLA-DRB1*04* increased the risk of developing high NAb titres, and in a subgroup analysis of *DRB1*04* alleles, the risk for NAbs was increased in *DRB1*04:01* carriers.
Conclusions: There is a preparation-specific genetically determined risk to develop NAbs against IFN-β. However, choice of IFN-β preparation still remains the single most significant determinant for the risk of developing NAbs.

WS4.5

Regulation of Autoimmunity by Reactive Oxygen Species in SKG Arthritis

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Introduction: To dissect the mechanisms that underlie autoimmune diseases, various rodent models have been developed. In collagen-induced arthritis (CIA), it was previously demonstrated that lack of reactive oxygen species (ROS) burst by phagocytes increases susceptibility to arthritis.

Method: To further investigate the role of ROS in autoimmunity, ROS-deficient Ncf1*/* mice were crossed with SKG mice. SKG mice are susceptible to chronic T cell-driven arthritis following activation of innate immunity by a single injection of mannan, a polysaccharide extracted from yeast. This susceptibility is caused by a point mutation in ZAP70 that decreases TCR signalling and thus alters thymic selection, leading to a more self-reactive T cells repertoire. Hence, this model does not rely on immunization with specific antigens, unlike in CIA, and therefore allows the study of ROS in spontaneous self-reactivity.

Results: The ROS deficiency made SKG mice more susceptible to arthritis. The disease onset and incidence were similar to SKG controls; however, the severity was much higher in SKG.Ncf1*/* mice especially in the chronic phase. Using *in vivo* imaging, a very high spike of ROS was detected in arthritic joints of regular SKG mice at the disease onset, which was then followed by a lower yet constantly higher level of ROS in comparison with wild type, which suggests a correlation between disease progression and ROS concentration in joints. However, SKG.Ncf1*/* mice, which have no detectable ROS production in joints, developed a much more severe disease. To better understand how ROS from phagocytes can limit arthritis severity in SKG mice cell and serum transfer experiments are under way, as well as thorough characterization of T cell and B cell responses.

Conclusion: Altogether, these results demonstrate the role of ROS in autoimmunity, which, as opposed to traditional belief, has a regulatory function by limiting tissue damage and improving clinical outcome.

WS4.6

Altered Phenotype and Stat1 Expression in TLR7/8-Stimulated Monocyte-Derived Dendritic Cells from Patients with Primary Sjögren's Syndrome

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Introduction: Dendritic cells (DC) are the most potent antigen-presenting cells of the immune system, involved in both initiating immune responses and maintaining tolerance. Dysfunctional and via toll-like receptor (TLR) ligands activated DC have been implicated in the development of autoimmune diseases. Therefore, we analysed the phenotype and functional properties of immature and TLR7/8-stimulated monocyte-derived DC (moDC) from patients with primary Sjögren's syndrome (pSS), a chronic inflammatory autoimmune disease characterized by progressive mononuclear cell infiltration in the exocrine glands.

Methods: The phenotype, apoptosis susceptibility and endocytic capacity of moDC were analysed by flow cytometry. Secretion of cytokines was measured by ELISA and multiplex analyses in moDC cell culture supernatants. The expression of TLR7 was analysed by flow cytometry and RT-PCR. Expression of Ro52/SSA, IRF-8, Bim, Stat1, p-Stat1 (Tyr701), p-Stat1 (Ser727), Stat3, pStat3 and GAPDH was measured by Western blotting. NF-kB family members (p50, p65, c-Rel, p52 and RelB) were quantified using the ELISA-based TransAM NF-kB family kit.

Results: We could not detect differences in expression of co-stimulatory molecules and maturation markers such as CD86, CD80, CD40 or CD83 on moDC from patients compared to healthy controls. Moreover, we could not observe variations in apoptosis susceptibility, Bim and Ro52 expression and the endocytic capacity of the moDC. However, we found that moDC from pSS patients expressed increased levels of the MHC class II molecule HLA-DR. We also found significant differences in cytokine production by moDC, whereas increased IL-12p40 secretion in mature pSS moDC correlated with increased RelB expression. Strikingly, moDC from pSS patients matured for 48 h with TLR7/8 ligand CL097 expressed significantly less Stat1.

Conclusion: Our results suggest a role for moDC in the pathogenesis of Sjögren's syndrome.

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WS4.7

B Cells and IgE in Type 1 Diabetes

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B cells contribute to the pathogenesis in type 1 diabetes (T1D), a chronic autoimmune disease in which the insulin-producing beta cells are destroyed leading to hyperglycaemia. Autoantibodies against β-cells precede disease development in susceptible individuals and are good diagnostic markers for screening patients. In addition, anti-CD20 treatment in prediabetic individuals has been shown to delay T1D onset. The non-obese diabetic (NOD) mouse is extensively used to study the pathogenesis of T1D and develops the disease in two phases – insulitis and overt diabetes, similar to humans. Previously, our laboratory has established that NOD B cells incongruously capture IgM and IgG molecules on the surface, promoting enhanced immune complex trapping. This has also been observed for IgE. IgE is usually associated with allergy. Interestingly, several studies have shown that presence of IgE autoantibodies in autoimmune patients, including T1D, is linked to poor disease prognosis. In our study, we aimed to elucidate the role/importance of IgE in T1D using the NOD mouse. Age-matched NOD and C57BL/6 (B6) female mice were used for the study. Flow cytometric analysis of splenic B cells from NOD and B6 revealed increased IgE levels on both CD23 high and CD23 dim NOD B cells, indicating that additional molecules can bind IgE. Immunohistochemical analysis of the spleen confirmed increased IgE level in NOD. Levels of IgE in the serum did not correlate with the surface levels on B cells as observed on an individual basis, possibly due to capture on B cell surface. IgE was also observed in the vicinity of the pancreatic islets, indicating its role in autoimmunity. However, the target for IgE is yet to be determined. We have planned to eliminate IgE from the NOD mouse system using anti-IgE to determine whether this leads to decreased T1D development.

WS4.8

Dysregulated Responses to Interferons in Autoimmune Addison's Disease

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Autoimmune Addison's disease (AAD), or primary adrenocortical insufficiency, is a classic organ-specific autoimmune disorder with poorly described pathogenesis. Genetic associations with HLA variants and immune responses against specific adrenal antigens point to a well-coordinated immunological attack on steroidogenic cells of the adrenal cortex, but the actual triggers of this attack remain completely unknown. In particular, knowledge about the roles played by innate immunity and the adrenocortical cells themselves is lacking. The interferon-inducible chemokine CXCL10 is elevated in the serum of patients with AAD and can also be induced at high levels in adrenocortical cells stimulated with interferons or pathogen-associated molecular patterns. However, it is also possible that this chemokine is produced by activated immune cells in the patients. In the present study, we have investigated whether peripheral blood mononuclear cells (PBMC) from AAD patients display an enhanced propensity to produce CXCL10 after direct or indirect stimulation with type I or -II interferons. Surprisingly, although serum levels of CXCL10 and CXCL9 were significantly higher in patients than in controls, patient PBMC produced significantly less CXCL10 and CXCL9 than healthy controls after stimulation with interferon-α, -β and -γ. This phenomenon was not evident when CXCL10 production was induced indirectly by stimulating PBMC with poly (I:C). We therefore conclude that PBMC from patients with AAD are deficient in their response to interferons. This might be related to their substitution therapy with glucocorticoids, but this should be investigated on the molecular level. Furthermore, the aforementioned observations suggest that the adrenal cortex itself is responsible for the increased serum levels of CXCL10 in the AAD patients.

WS4.9

CCL2 Chemokine in Central Tolerance

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CCL2 is an inflammation-associated chemokine that induces the recruitment of immune cells in tissues. CCL2 and its receptor CCR2 are implicated in promoting experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. However, we observed that transgenic mice expressing CCL2 in central nervous system (MBP/CCL2) were resistant to EAE. MBP/CCL2 mice crossed with 2D2 mice, whose T cells express a transgenic T cell receptors (TCR) specific for the self-antigen myelin oligodendrocyte glycoprotein (MOG), were also resistant to EAE. Both MBP/CCL2 and 2D2 × CCL2 mice showed ectopic expression of CCL2 in thymus.

T cell development is guided by interactions with thymic stromal cells. Among them, dendritic cells (DC) delete or anergize $CD4^+$ and $CD8^+$ thymocytes whose TCR recognize a complex of self-peptide + major histocompatibility complex, so that only T cells tolerant to self-peptides leave the thymus. Three subsets of DC have been identified in mouse thymus. They all express CCR2 and two of the DC subtypes can take up antigens in periphery and migrate into the thymus to mediate tolerance.

We thus investigated whether thymic expression of CCL2 could tolerize encephalitogenic T cells and inhibit EAE development.

We showed that ectopic expression of CCL2 in thymus of MOG-TCR transgenic mice induced a massive deletion of MOG-specific T cells. CD4-expressing MOG-specific T cells were not detected in thymus or lymph nodes. Thymus weight and thymus cell number were also strongly decreased. The ectopic expression of CCL2 in thymus was accompanied by increased number of plasmacytoid DC (pDC) in thymus and we showed that pDC expressed CCR2. We also identified stromal cells that constitutively expressed CCL2 in B6 mouse thymus.

Based on these results, we propose that overexpression of CCL2 in thymus recruits pDC which delete encephalitogenic T cells, thereby inhibiting EAE development. This identifies a novel role for CCL2 in T cell tolerance.

WS4.10

Profiling of Intestinal Microbiota of Paediatric Patients After Stem Cell Transplantation

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Background: Gastrointestinal complications constitute a major problem in allogeneic haematopoietic stem cell transplantation (HSCT). Intensive pretransplantation conditioning contributes to intestinal complications by disrupting the integrity of the intestinal epithelium. This can lead to translocation of intestinal microbes and microbial structures into tissues. Lipopolysaccharide (LPS) of Gram-negative bacteria is a strong inducer of pro-inflammatory cytokines and found in serum in graft-versus-host disease (GvHD), which is a severe immunological complication of HSCT and also contributes to damage of intestinal mucosa. Intestinal microbiota in healthy individuals is highly diverse and estimated to consist of at least 1000 different bacterial species. In our ongoing study, we follow the composition of intestinal microbiota in paediatric patients after HSCT. We also study roles of immunological responses towards microbial structures in transplantation complications.

Methods: From faeces samples collected before and during a 6-month period after transplantation, microbial profiles have been analysed by PCR-DGGE (denaturing gradient gel electrophoresis) and by 16S rRNA pyrosequencing. We have also measured LPS-induced cytokine production from peripheral blood mononuclear cells (PBMC) obtained from sibling donors of the grafts.

Results and conclusions: Preliminary results indicate a significant disturbance of the intestinal microbiota in many patients. This was seen as high dominance of single bacterial genera following the immediate post-transplant phase. Recovery of microbiota, indicated by re-appearance of common intestinal bacterial genera and increased diversity of microbiota, could be seen 4–6 months post-transplantation. The results suggest that profiling of intestinal microbiota can be a useful tool in patient follow-up after transplantation.

WS4.11

Ro52/TRIM21 Expression is Decreased in Malignant B Cells

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Introduction: TRIM21 (Ro52) is a rheumatic autoantigen predominantly expressed in leucocytes. Several members of the TRIM (tripartite motif) protein family regulate basic cellular processes, including proliferation and apoptosis. Interestingly, high expression of TRIM21 is associated with decreased proliferation and increased apoptosis *in vitro* and the *Trim21* gene is located in a tumour suppressor locus. Rheumatic patients are prone to developing B cell lymphomas, especially diffuse large B cell lymphoma (DLBCL). Hypothesizing that TRIM21 expression may be altered in lymphoma cells, we investigated TRIM21 expression in tumour tissue and several human cell lines.

Methods: Lymphoma biopsies obtained from patients with DLBCL with and without a history of rheumatic disease were investigated for TRIM21 expression by immunohistochemistry using two different TRIM21 monoclonal antibodies. TRIM21 mRNA contents was analysed by qPCR in six human B cell lines (FLEB14-4, KM3, Daudi, Raji, U266 and NALM-6), a T cell line (Jurkat) and $CD19^+$ B cells from a healthy donor.

Results: We observed that loss of TRIM21 expression in lymphoma tissue significantly correlated with shorter

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overall and progression-free survival in DLBCL patients. This association was independent of rheumatic disease and autoantibody status. TRIM21 mRNA expression was considerably lower in all investigated B cell lymphoma lines as well as Jurkat cells (0,3–4,8 relative normalized expression units) compared to normal human B lymphocytes (7,1 relative normalized expression units).

Conclusion: Our data indicate that TRIM21 expression is deficient in both DLBCL and B cell lymphoma-derived cell lines compared to normal lymphoid cells. Further, TRIM21 expression associates with a more aggressive course of lymphoma. Analysis of TRIM21 expression in DLBCL may thus have an additional value in clinical assessments of the prognosis.

WS4.12

Profiling of Peripheral Blood Cells in Patients with Rheumatic Diseases Using TruCount Technology

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Objectives: We analysed the cellular profile of peripheral blood leucocytes in patients with three different autoimmune diseases, primary Sjögren's syndrome (pSS), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), and healthy controls, to determine whether this could be used to differentiate between patient groups and to get more insight into the pathogenesis of these diseases.

Methods: Peripheral blood from healthy controls (n = 20), patients with pSS (n = 27), SLE (n = 13) and RA (n = 14) was collected into heparin tubes, and 50 µl was transferred to a TruCount tube (BD Bioscience), containing titrated amounts of antibodies, to determine the absolute number of different leucocyte populations. After 15-min incubation, erythrocyte lysis buffer was added to remove red blood cells. The cells were analysed within 4 h on a BDFortessa flow cytometer (BD Bioscience). Antibodies used were CD45 Qdot 605, CD3 Horizon V500, CD56 PE-Cy7, CD4 Pacific blue, CD20 Alexa Fluor 647, CD8 PerCP-Cy5.5. Analyses were performed using FlowJo software (Treestar).

Results: The cellular profile of peripheral blood of the different patient groups varied significantly from each other and the healthy controls. Patients with RA had significantly increased amounts of granulocytes and monocytes compared to healthy controls, while the amount of lymphocytes was similar in all analysed groups. SLE patients had significantly reduced numbers of CD20+ B cells compared to healthy controls. The number of CD3+ T cells and CD8+ T cells was similar in all subgroups, but CD4+ T cells were significantly increased in RA patients compared to SLE patients. CD56+ NK cells were significantly increased in RA patients compared to patients with pSS and SLE.

Conclusion: TruCount analyses resulted in distinct cellular profiles of peripheral blood leucocytes in the three analysed patient groups and might help to understand underlying defects in these autoimmune diseases.

WS4.13

Characterization of the Sjögren's Syndrome Susceptibility Genes Fam167/Blk

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Sjögren's syndrome is a common autoimmune disease, primarily affecting exocrine glands, particularly salivary and lacrimal glands. Sjögren's syndrome primarily involves B cells which show defective differentiation patterns resulting in altered activation and trafficking. A genome-wide association study of genetic polymorphisms in Sjögren's syndrome identified six novel non-HLA gene loci as associated with Sjögren's syndrome. The FAM167A/BLK locus on chromosome 8 was one of the identified loci, with a $P < 5 \times 10^{-10}$ for the most significant SNP. The Sjögren's associated genotype in the FAM167A/BLK locus regulated increased expression of the FAM167A gene and decreased expression of BLK. BLK functions in signalling downstream of the B cell receptor, while FAM167A has no known function.

To begin understanding the function of fam167a, the expression pattern of fam167a was characterized in organ samples collected from C57BL/6 mice, including skin, brain, lung, salivary gland, lymph node, thymus, heart, skeletal muscle, intestine, kidney, spleen and liver. Lymphocyte subtypes sorted from lymph nodes, spleen and thymus from mice were also collected. RNA was isolated using an RNeasy isolation kit (Qiagen), cDNA was generated using iScript (BioRad), and the presence of fam167a expression was measured in comparison with housekeeping genes (GAPDH and HPRT) using a SYBR green-based qPCR. To assess the intracellular localization of fam167a, the gene was cloned using the Gateway system (Invitrogen) and a YFP-tagged expression clone transfected into 3T3 cells.

Mouse skin, lungs, spleen and lymph nodes all showed expression of fam167a, as well as sorted double negative T cells ($CD3^+CD4^-CD8^-$) and marginal zone B cells. After transfection of YFP-tagged fam167a, punctuate dot-like cytoplasmic fluorescence was observed in 3T3 cells. Fam167a thus appears to have a potentially cytoplasmic function in lymphocytes and epithelium.

WS4.14

Cardiopathogenic Role of Human RO/SSA and LA/SSA Antibodies in a Novel Animal Model of Congenital Heart Block

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Background: Congenital heart block (CHB) develops in children of Ro/SSA- and/or La/SSB-positive mothers. However, the mechanism behind the disease remains unknown.

Objectives: To study the effect on cardiac conduction of purified human IgG antibodies from a CHB patient mother in an animal model.

Methods: Pregnant adult female Dark Agouti rats (Charles-Rivers, Germany) were injected intraperitoneally with 4 mg of IgG purified from a CHB anti-Ro52-p200 antibody-positive mother, a healthy control or vehicle. The assessment of foetal cardiac function during pregnancy was performed by foetal echocardiography/Doppler recordings. Neonatal cardiac function after birth was assessed by ECG recordings within 24 h after birth.

Results: Performing echocardiography/Doppler, we observed significant bradycardia and AV time prolongation in the group injected with 4 mg patient IgG compared to other groups ($P = 0.0004$; $P = 0.001$) as well as a significant prolongation of isovolumetric contraction time (ICT) and ejection time (ET) time ($P = 0.01$; $P = 0.007$). Furthermore, pups in the group injected with patient IgG had a significantly higher myocardial performance index (MPI; $P = 0.002$) than in the group injected with control IgG.

The ECG recordings showed that the pups from the group injected with patient IgG have a significant reduction in mean heart rate ($P < 0.0001$) as well as a significant prolongation of the PR and RR intervals compared to the group injected with IgG control sera ($P < 0.0001$).

Conclusions: Our data suggest that maternal autoantibodies induce foetal bradycardia, AV time prolongation and decrease cardiac performance, mimicking features of the human disease and confirming a role for the autoantibodies in disease pathogenesis. The data also suggest that an animal model for CHB can be established by a simple technique of passive transfer of human IgG from a mother with a child with CHB. This model will be highly relevant for investigating the pathogenesis of CHB and evaluating novel therapeutic approaches and drugs.

WS4.15

Evidence of Marginal Zone B Cells in the Lymph Nodes Presenting Autoantigen to T Cells in a Model of Autoimmune Arthritis

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B cells play a major role in the pathogenesis of many autoimmune disorders, including rheumatoid arthritis (RA). However, it is not fully known how the B cells contribute to arthritis. A significant fraction of B cells in the peripheral repertoire express self-reactivity, and many of these B cells belong to innate-like subsets such as the marginal zone (MZ) B cells. We show here that MZ B cells in the spleen have natural self-reactivity to type II collagen (CII) and initiate an early response to CII during the induction of collagen-induced arthritis, a mouse model for RA. Furthermore, the MZ B cells were very potent CII-presenting cells inducing proliferation of CII-specific T cells *ex vivo*. Notably, an expansion of a small MZ B cell-like (MZBL) population was also evident in the lymph nodes following immunization with CII but not with a control protein or the adjuvant alone. The MZBL cells displayed MZ B cell characteristics and had an antigen-activated phenotype with high surface expression of CD80 and CD86 and could present CII to T cells.

We show here that self-reactive MZ B cells that are retained in the repertoire can be activated in the spleen and lymph nodes to promote autoimmune arthritis by breaking T cell tolerance. This may then allow autoreactive T cells to assist follicular B cells in the production of pathogenic autoantibodies.

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WS4.16

The Telomeric End of MHC Class III Regulates Experimental Arthritis in Rats

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Rheumatoid arthritis (RA) is a multifactorial, chronic inflammatory joint disease with the strongest association in the HLA-DRB1 locus within the major histocompatibility complex (MHC). In addition to the DRB1 locus, human genetic association studies have suggested the disease association of MHC class III region. However, MHC class III is a region difficult to study in human due to its high gene density and strong linkage disequilibrium. MHC class III contains many candidate genes in RA, including tumour necrosis factor (TNF), lymphotoxin-alpha (LTa) and allograft-inflammatory factor (AIF-1).

Previous linkage analysis showed the association of MHC *Pia1* with both pristane-induced arthritis (PIA) and oil-induced arthritis (OIA) in rats. Disease-phenotyping intra-MHC recombinant inbred strains covering different parts of MHC revealed two arthritis regulation loci within the *Pia1* locus, one in MHC class II and one in MHC class III. For MHC class III, we have further mapped arthritis susceptibility to a minimal interval of around 40–60 kb in the telomeric end of MHC class III. Compared to the DA rats control, the MHC class III congenic rats developed less severe PIA and OIA with less weight loss and a later day of disease onset, and also less damaged joints with significantly less bone erosion and cartilage destruction. Adoptive transfer of oil-primed T cells showed that the locus controlled immune priming of the disease. Furthermore, quantitative real-time PCR revealed the differential expression of two genes in the region, and sequence analysis showed a number of genetic variants in the region which could be important in understanding the disease regulation.

In conclusion, we showed that telomeric end of MHC class III regulates experimental arthritis and controls immune priming of arthritis in rats. Identification of the disease regulation gene in MHC class III may provide novel target in treatment aimed at reducing disease severity and joint damage.

WS4.17

Pregnancy Induces a Reduction of CCL25 and CXCL12 Expression in Thymic Cortical Epithelial Cells and Fibroblasts

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During normal pregnancy, the thymus undergoes severe reduction in size and thymocyte output, which may contribute to the maternal–foetal tolerance. The mechanisms behind this pregnancy-induced thymic involution, however, are presently unknown. The aim of the study was to characterize changes in different thymocyte subpopulations in pregnancy to detect a possible block in their development. In addition, the study aimed to determine pregnancy-induced changes in thymic chemokine production by thymic stromal cells to determine a possible defect in seeding of the thymocyte progenitors.

Thymocyte subpopulations were analysed by FACS in mice thymi at three time points of normal pregnancy. In addition, the thymic medullary epithelial cells, cortical epithelial cells, fibroblasts and dendritic cells were FACS-sorted, followed by the expression analysis by qPCR for the key chemokines behind the seeding of thymocyte progenitors.

Although pregnancy induced a progressive reduction in thymocyte numbers, the proportion of their major subpopulations remained unchanged. However, in cortical epithelial cells and thymic fibroblasts, pregnancy induced a substantial reduction in the expression level of CCL25 and CXCL12.

The data indicate a role for cortical epithelial cells and fibroblasts, which via reduced expression of CCL25 and CXCL12 may play a role in the pregnancy-induced thymic involution. The resulting changes in recruitment of thymocyte progenitors and sequential changes in thymic output may contribute to the immune tolerance in pregnancy.

WS4.18

Thymic Export of T Cells During Pregnancy: Can Differential Regulation of T Cell Populations Contribute to Foetal Tolerance?

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Despite the prominent role of thymus in T cell-mediated immunity, there is little knowledge regarding its function in human pregnancy and pregnancy-associated tolerance towards the semi-allogenic foetus. In this exploratory study, we investigated the function of thymus during pregnancy by analysing the output of T helper cells from thymus. DNA from isolated CD4+ blood cells from 26 healthy second-trimester pregnant and 20 age-matched non-pregnant women were analysed for the content of T cell receptor excision circles (TRECs), which are enriched in newly synthesized T cells. In addition, the frequency of recent thymic emigrant (RTE) T helper subsets was assessed in whole blood from pregnant (n = 10) and non-pregnant women (n = 8) by flow cytometry, utilizing CD31 and CD45RA as markers of recent thymic exposure and naïve phenotype, respectively. Pregnant women showed significantly reduced levels of TREC in the CD4+ population as compared to non-pregnant women (P = 0.041), which indicates a reduced output of CD4+ T helper cells from the thymus, being in line with the observed shrinkage of thymus in mouse pregnancy. Further, we attempted to determine whether the output of the regulatory T cell (Treg) subpopulation was also decreased during pregnancy or whether it was differentially regulated. Preliminary results suggest an increased thymic output of Tregs during pregnancy as measured by the frequency of CD31+CD45RA+Tregs (P = 0.018). In conclusion, we show reduced TREC levels in CD4+ cells in pregnant women, likely reflecting a reduced output of the whole T helper cell population from thymus which could be of importance for limiting the entry of potential allogenic antifoetal T cells into the periphery. However, the output of Tregs may be differentially regulated, thereby further contributing to the establishment and maintenance of the foetal-tolerant environment required for successful pregnancy.

WS4.19

Alternative Splicing of FOXP3 is Associated with Atherosclerotic Plaque Instability

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Atherosclerosis, a chronic inflammatory disease of arteries, is the leading cause of cardiovascular diseases. Atherosclerosis is initiated when low-density lipoprotein is retained in the subendothelial space of arteries resulting in pathological immune responses and formation of an atherosclerotic plaque. The main clinical complication occurs when a plaque ruptures causing formation of a thrombus that can further induce life-threatening vascular events. Previous studies have demonstrated that effector T cells contribute to atherogenesis. In contrast, it has become clear that immunosuppressive CD4+FOXP3+ regulatory T (TReg) cells have an atheroprotective role in experimental models of atherosclerosis. Yet, it remains to be determined how TRegs modulate clinical atherosclerosis. The transcription factor Forkhead box P3 (FOXP3) protein defines the lineage of TRegs and plays a critical role in upholding their suppressive activity. Three splice forms of *F0XP3* mRNA exist in humans, which gives rise to protein isoforms with different functional properties. Full-length FOXP3 and FOXP3d2 confer a suppressive phenotype to TReg cells, whereas FOXP3d2d7 inhibits the function of the other FOXP3 isoforms in a dominant negative manner. Herein, we have analysed *F0XP3* splice variants expression in more than 200 blood samples and 200 atherosclerotic plaques in relation to plaque stability and effect of HMG-CoA reductase inhibitors treatment. We have observed that while the total amount of *F0XP3* transcripts stay stable between symptomatic versus asymptomatic plaques, there is a shift in the isoform ratio where relatively low levels of FOXP3d2 are found in symptomatic plaques.

It is well established that TReg cells have the capacity to suppress immune responses. However, in order to harness their full clinical potential, we need to fully understand how they function. Our results highlight the importance of characterizing FOXP3 expression on an isoform basis and suggest that immune responses may be manipulated by modulating the expression of FOXP3 isoforms.

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WS4.20

Amino Acids Situated in NLS Domain of AIRE Protein are Required for DNA Binding

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The autoimmune regulator (AIRE) is a transcriptional regulator expressed in a specific subset of thymic epithelial cells and promoting activation of thousands genes there. It is established that AIRE influences transcription in elongation stage by releasing stalled RNA polymerase. Despite many exciting hypotheses, the recruitment of AIRE to its target genes and the mechanism of genes to be activated are not completely understood at the moment.
The interaction of PHD1 domain of AIRE with non-methylated H3 was demonstrated in previous years and its importance for target gene activation by AIRE was shown. In our experiments, we observed even stronger binding of AIRE to episomal plasmids than to chromatin despite low histone content of plasmids. There are different reports about SAND domain role in mediating DNA binding of AIRE as well as DNA-PK participation in this process. We performed EMSA and agarose gel shift assays to determine that part of NLS domain is responsible for AIRE binding to oligos and plasmids. Our ChIP experiments with specific amino acids mutated confirmed our finding that amino acids situated in NLS domain but not crucial for nuclear localization are responsible for binding to plasmids. The dimerization of AIRE through CARD domain is also important for DNA binding as the mutations disrupting dimerization prevent also binding in ChIP experiments. There is possible interplay between domains in AIRE recruitment with NLS playing major role in the case of more open DNA and PHD1 taking over in the case of chromatin coated with histones.

WS4.21

Characteristics of T Cell Subsets in Smokers and Multiple Sclerosis Patients' Lungs

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Background: Smoking has been shown to be a considerable risk factor for various autoimmune diseases, especially in combination with certain genetic variants. With regard to multiple sclerosis (MS), a recent study found a strong gene–environment interaction, because expression of HLA-DRB1*15 and absence of HLA-A*02 was associated with a 16-fold increase in risk of disease. Recent studies in the EAE model of MS have further shown that prior to entering the CNS, T cells migrate to the lung, where extensive activation and reprogramming of autoreactive T cells occur in the lung-associated lymphoid tissues. This indicates that important immunological events in the lungs could contribute in a regulatory manner, as a prerequisite for autoreactive T cells to enter their target tissues and induce autoimmunity.
Methods: Bronchoalveolar lavage (BAL) fluid and peripheral blood from healthy non-smoking controls, healthy smokers and MS patients (smoking and non-smoking/ex-smokers) with normal lung function are obtained for this ongoing study. *Ex vivo* investigation of samples is carried out by staining with panels of antibodies against selectively chosen activation/regulatory/migratory markers followed by flow cytometric analysis.
Results: Preliminary data from healthy non-smoking controls ($N = 7$), smokers ($N = 10$) and MS non-smoking patients ($N = 8$) and MS smoking patients ($N = 3$) show increased expression of the activation marker CD40L (suggesting recent antigen encounter) on CD4+ T cells from MS BAL fluid compared to BAL fluid from smoker sand non-smokers. Proliferation of CD4+ and CD8+ T cells in MS BAL fluid as well as smoking controls is also higher, than non-smoking patients. Additionally, BAL samples from MS patients, smoking and non-smoking, have higher frequencies of Helios-positive CD4+CD25+ and CD4+FOXP3+ cells compared to healthy smokers and non-smokers.
Conclusion: From the preliminary data, it seems there are interesting differences between the various subject groups. It is, however, too early to make full conclusions as the study is ongoing.

WS4.22

Importance of Indirect Immunofluorescence Methodology in Antinuclear Antibody Screening

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Introduction: The importance of antinuclear antibody (ANA) screening in diagnostic workup of autoimmune disease is well known. The rate of positive screening is about 0.5% in the general population and up to 90% in patients with systemic lupus erythematosus (SLE). Today several methodologies are commonly used for ANA screening. The methodology recommended by the ACR and EULAR is indirect immunofluorescence (IIF). However, some laboratories use ENA-ELISA for the same purpose. The aim of this study was to evaluate the strength of the above methodologies in the routine use of ANA in Iceland.

Methods: The rate of positive ANA test was evaluated for 1-year time. We evaluated these different methodologies used for ANA screening including IIF on rodent tissue and HEp-2000 cells and ENA-ELISA. All samples that were ENA positive were evaluated for specific ENA profile (Sm, nRNP, SSA, SSB, Scl-70, Jo-1 and dsDNA).

Results: For a 1-year time, 3556 samples were evaluated of whom 42.4% were positive. Of the ANA positive, only 12.6% were ENA positive. To evaluate the strength of the various ANA screening methods, we analysed additional 196 samples further. Of the 196 samples, 79.6% were positive in ANA screen test on rodent tissue and 91.3% on HEp-2000 cells. On rodent tissue, 25% had positive ENA profile and 22.3% on HEp-2000 cells. Out of the samples that were ANA positive and ENA negative, 82.9% on rodent tissue and 79.5% on HEp-2000 cells had joint pain or arthritis. From these ANA-positive and ENA-negative samples, 21% had been diagnosed of SLE, 20% with rheumatoid arthritis and about 30% were strongly suspected to suffer from other autoimmune diseases.

Conclusion: The results clearly demonstrate the importance of IIF methodologies in ANA screening, given their significant accretion with an underlying autoimmune disease even in ENA-negative individuals.

WS4.23

CD8+ T Cells, Not HLA Class II Risk Allele–Restricted CD4+ T Cells, Mediate Transfer Autoimmune Pancreatitis in HLA-DRB1*0405 Transgenic Ab0 Rag1−/− NOD Mice

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Background: Autoimmune diseases are frequently associated with specific HLA class II risk alleles. The isolation from autoimmune disease patients of risk allele-restricted, self-antigen-specific CD4+ effector T cells suggests that pathogenic T cells may recognize self-antigens presented on HLA class II risk alleles. This mechanism could lead to CD4+ T cell activation, inflammatory cytokine secretion and organ damage in autoimmune diseases.

Autoimmune pancreatitis (AIP) in humans is associated with the HLA-DRB1*0405/DQB1*0401 haplotype. We recently described a model of AIP in HLA-DRB1*0405 transgenic Ab0 NOD mice (Freitag TL et al.; Gastroenterology 2010). Contrary to HLA class II transgenic control strains, these mice develop spontaneous AIP, characterized by destructive infiltration of the exocrine pancreas with CD4+ and CD8+ T cells, B cells and macrophages.

Objectives: To identify the pathogenic lymphocyte population causing AIP in HLA-DRB1*0405-transgenic Ab0 NOD mice.

Methods: T and B cell-deficient, HLA-DRB1*0405 transgenic Ab0 Rag1−/− NOD recipient mice were adoptively transferred with CD4+ T cells, CD8+ T cells or CD19+ B cells i.v., from HLA-DRB1*0405/huCD4 transgenic Ab0 NOD donor mice. Recipients were monitored for up to 16 weeks post-transfer.

Results: Only adoptive transfers with CD8+ T cells, but not with CD4+ T cells or CD19+ B cells, resulted in AIP and pancreatic atrophy in recipient mice. This was associated with significant body weight loss and reductions in pancreas weight versus controls.

Conclusions: CD8+ T cell transfer, not HLA class II-restricted CD4+ T cell transfer, causes AIP in HLA-DRB1*0405-transgenic Ab0 Rag1−/− NOD mice. This suggests that in immunocompetent mice HLA class II risk allele expression fails to protect from CD8+ T cell-mediated AIP, likely due to defects in organ-specific self-tolerance maintained by regulatory CD4+ T cells (Treg). This model can be used to explore the functional roles of HLA class II alleles in human autoimmune diseases.

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WS4.24

Autoantibodies Against Ganglioside GM3 are Associated with Narcolepsy–Cataplexy Developing After Pandemrix® Vaccination Against 2009 Pandemic H1N1-Type Influenza Virus

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After the mass vaccinations against the pandemic H1N1 influenza virus, a sudden increase in juvenile narcolepsy was reported in six European countries where AS03-adjuvanted Pandemrix® vaccine was used.

Narcolepsy with cataplexy (NC) is a chronic neurological disease characterized by an excessive daytime sleepiness, hallucinations and cataplexy. In NC, the hypocretin 1-producing neurons in the hypothalamus are destroyed, most probably by an autoimmune reaction. Both genetic (e.g. HLA-DQB1*0602) and environmental risk factors (e.g. Pandemrix®) contribute to the disease development, but the underlying autoimmune mechanisms are largely unknown.

Because influenza virus haemagglutinin is known to bind gangliosides, which serve as virus receptors, we screened the sera of NC patients and controls for IgG antiganglioside antibodies (AGAs) against 11 human brain gangliosides (GM1, GM2, GM3, GM4, GD1a, GD1b, GD2, GD3, GT1a, GT1b, GQ1b) and a sulfatide. Samples from 173 children were analysed: 48 with post-Pandemrix NC, 20 with NC without Pandemrix® association, 57 Pandemrix-vaccinated and 48 unvaccinated children. We found that anti-GM3 antibodies were significantly associated ($P < 0.05$) with post-Pandemrix NC (15% of patients versus 4% of vaccinated controls). In general, AGAs were more frequent in vaccinated (18%) than in unvaccinated (7%) individuals. Our data suggest that autoimmunity against GM3 is a feature of Pandemrix®-triggered NC and autoantibodies against gangliosides were induced by Pandemrix® vaccination. Thus, in addition to a cell-mediated immune response, post-vaccination narcolepsy involves humoral autoimmunity.

WS4.25

Identification of a RANKL-Specific Gene Set in Stimulated Foetal Thymic Organ Culture

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Medullary thymic epithelial cells (mTECs) express a large variety of tissue-specific antigens and present them to developing lymphocytes during the process of thymic negative selection, thus preventing autoimmunity through the regulation of T cell development. Mature mTECs are crucial for proper induction of central tolerance, which is why many recent studies have concentrated on cellular signals driving mTEC maturation. It has been shown that several receptors and ligands of the TNF superfamily (TNFSF) are needed for proper mTEC development. However, knowledge of their exact roles in regulating gene expression of thymic epithelial cells is rather limited. The aims of this study were to identify genes induced by RANK, TNFR1, CD40 and LTβR signalling in the thymus and in particular to identify genes specifically induced by RANK-RANKL signalling, due to its key role in mTEC maturation. Thymic lobes of embryonic day 16.5 mice were removed and cultured for 6 days in the presence of 2′-deoxyguanosine to deplete thymocytes. Thereafter, the lobes were stimulated with selected TNFSF members for 48 h. Material was collected and analysed using Illumina BeadChip expression array to assess changes in transcript levels. Selected genes were validated using qPCR.

We found that different TNFSF ligands induced the expression of tens to hundreds of genes, including Aire, tissue-specific antigens and many thymic chemokines. We observed largely overlapping lists of genes regulated by various ligands, which suggests that single gene defects can be compensated for by other signals and that a complex regulatory network exists for target gene expression. However, we identified 15 genes induced specifically by RANKL, which, considering the central role of RANK signalling in maintaining the thymic microenvironment, are likely to play an important role in mTEC maturation and regulate the induction of central tolerance.

WS4.26

Antibodies Against Carbamylated Proteins in Serum from Patients with Primary Sjögren's Syndrome

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Sjögren's syndrome (SS) is an autoimmune rheumatic disease with organ-specific features, characterized by the infiltration of mononuclear cells in the exocrine glands, primarily the salivary and lachrymal glands. The presence of autoantibodies towards intracellular Ro/SSA and La/SSB antigens is commonly found and serves as diagnostic criteria. In this study, we sought to evaluate the presence of antibodies against carbamylated proteins (anti-CarP) in patients with primary Sjögren's syndrome (pSS).

Sera from 84 patients with pSS recruited from the Rheumatology Clinic at the Haukeland University Hospital in Bergen, Norway, and 82 gender- and age-matched healthy controls, recruited from the same geographical area through the Haukeland University hospital Blood Bank, were used in this study. All pSS patients fulfilled the American-European Consensus criteria (AECC) for the classification of SS. Samples were analysed for the presence of IgG antibodies against carbamylated FCS by ELISA.

pSS patients had significantly increased level of aCarP antibodies in the serum as compared to healthy controls. Our results show that 26% of the serum of pSS patients and 7% of healthy controls analysed were positive for IgG anticarbamylated protein antibodies.

The reactivity against modified protein was over three times more frequent in patients as compared to healthy controls, indicating that the presence of anticarbamylated protein antibodies may serve as an additional predictive or diagnostic marker for Sjögren's syndrome.

WS4.27

Sex-Biased Expression of Sjögren's Syndrome Susceptibility Genes

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Introduction: Sjögren's syndrome is a chronic autoimmune disorder that mainly affects exocrine glands such as lacrimal and salivary glands. The disease predominantly affects women with a female-to-male ratio of 9–1, but the molecular mechanisms that underlie this sex bias are not well understood. Polymorphisms in several susceptibility loci that associate with Sjögren's syndrome have been recently identified, including the MHC region, IRF5, STAT4, IL12A, FAM167A/BLK and TNIP1 genes. In this study, we investigated whether the expression of Sjögren's syndrome susceptibility genes is sex specific at steady state by comparing expression in female and male mouse immune cells.

Methods: Spleen and lymph nodes were recovered from 15 male and 15 female C57BL7/6 mice of 10 weeks of age. RNA was extracted from the organ samples and cDNA was obtained by reverse transcriptase conversion. Quantitative PCR was performed for all samples, and expression levels were normalized with GAPDH as reference gene.

Results: The expression of five genes with the most significant association with Sjögren's syndrome was studied: IRF5, STAT4, IL12A, FAM167A, TNIP1 and BLK. The analysis showed that the expression levels were not significantly different between male and female mice at steady state. Further, there was no substantial difference in expression levels between the two tissues.

Conclusion: The sex bias observed in autoimmunity may not be attributed exclusively to differential gene expression at steady state. Rather, the molecular mechanisms regulating the expression of these genes in specific triggering situations might account for the sex skewing.

WS4.28

Identification of Molecules Targeted by Ro52 Autoantibodies in the Development of Congenital Heart Block

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Introduction: Congenital heart block (CHB) is associated with maternal anti-Ro52 autoantibodies, which are transported into the foetal circulation through the placenta and lead to a block in the cardiac signal conduction at the atrioventricular node. The mothers of affected infants are often diagnosed with autoimmune disease such as Sjögren's syndrome or systemic lupus erythematosus. As CHB-inducing antibodies bind the cardiomyocyte cell surface, while Ro52 is an intracellular protein which has no or little cardiac expression, it has been suggested that the maternal autoantibodies bind a cross-reactive cell surface molecule exposed in the foetal heart. The aim of the current study was to establish methodology for the identification and confirmation of cross-reactive targets of the Ro52 autoantibodies.

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Methods: Multiplexed antibody-based protein profiling and screening of protein components developed within the framework of the human protein atlas project (PrESTs) were performed for two mouse monoclonal antibodies that induce CHB in an experimental transfer model and one isotype-matched control monoclonal.

Results: An initial study with an 11,521 k PrEST array detected 13 proteins bound by the CHB-inducing monoclonal Ro52 antibodies, but not the control antibody. One PrEST, derived from the central (coiled-coil) part of integrin-linked kinase (ILK) protein, was bound by both CHB-inducing antibodies.

Conclusion: In conclusion, PrEST arrays could be an auxiliary tool for screening and identifying cross-reactive targets for normal and diseased states.

Workshop 5. Allergy/Tumour Immunology/Novel Concepts and Technologies

WS5.1

The Role of Microfibrillar-Associated Protein 4 in the Murine Model of Asthma

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Microfibrillar-associated protein 4 (MFAP4) is a matricellular protein abundant in lungs. Its functions remain largely unknown. However, due to the presence of an integrin-binding site and structural similarities to many proteins engaged in innate immune defences, MFAP4 is thought to be an active modulator of immune response and tissue remodelling.

The aim of the current study was to investigate a potential role of MFAP4 in two distinct murine models of allergic asthma.

BALB/c WT and MFAP4-deficient mice were sensitized and subsequently challenged intranasally with ovalbumin, or chronically challenged with house dust mite (HDM) extract. Lung function measurements and airway hyperresponsiveness (AHR) assessment were performed. Immune cell infiltration and cytokine production were measured in bronchoalveolar lavage (BAL) and lung tissue. Lungs were processed for histology and evaluated for signs of inflammation and airway remodelling.

In both models, treated MFAP4-deficient mice exhibited lowered cell counts in BAL than the WT littermates, corresponding to significantly diminished numbers of infiltrating eosinophils. Accordingly, the production of eosinophil chemoattractants CCL11 and CCL24 was reduced in the airways of MFAP4-deficient mice. Moreover, MFAP4-deficient mice were partially protected from allergy-induced mucus production, subepithelial fibrosis and smooth muscle cell hyperplasia. Lack of MFAP4 in chronically treated mice resulted in increased central airway resistance, a representative for tracheal stiffness.

Obtained results show that absence of MFAP4 attenuates allergy-induced airway remodelling and eosinophilia, but contributes to stiffness in conducting airways. It indicates that MFAP4 may serve as a potential therapeutic target in the treatment for allergic asthma.

WS5.2

Does Cell Proliferation Contribute to the OVA-Induced Increase of Lung Mast Cell Progenitors in a Mouse Asthma Model?

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Introduction: In patients with allergic asthma, mast cell numbers are elevated in the lung. The increase in mast cells can be recapitulated in experimental mouse models of asthma. Earlier studies using an ovalbumin (OVA) model of experimental asthma suggest that the increase in mast cells is due to the recruitment of mast cell progenitors from blood into the lung tissue. However, to what extent proliferation of lung-resident mast cell progenitors is contributing to the increase is currently unknown.

Methods: An experimental asthma model in which mice were sensitized i.p. on day 0 and day 7 followed by OVA aerosol challenge on days 17–19 was used. On day 20, mononuclear cells were isolated from the lung following enzymatic degradation and gradient centrifugation. Isolated single cell suspensions were stained with antibodies towards lineage markers, c-kit, ST2, integrin β7 and CD16/32 to identify mast cell progenitors. In addition, intracellular staining with the Ki-67 antibody to mark proliferating cells was performed.

Results: OVA sensitization and challenge increased the frequency and total number of lung mast cell progenitors fivefold over only sensitized mice. Approximately 45% of mast cell progenitors were Ki-67$^+$ in OVA-sensitized mice. After OVA sensitization and challenge, the percentage of Ki-67$^+$ mast cell progenitors was increased to about 70% corresponding to an eightfold increase in total number of proliferating mast cell progenitors.

Conclusions: Our data suggest that *in situ* cell proliferation may add to remarkable increase in lung mast cell progenitors.

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WS5.3

Insect Bite Hypersensitivity of Horses: Induction of a Specific Antibody Response in Horses After Oral Treatment with Transgenic Barley Grain Expressing an Insect Allergen

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Background: Insect bite hypersensitivity (IBH) is an IgE-mediated dermatitis of horses initiated by bites of midges of the genus *Culicoides* that do not exist in Iceland. The prevalence of IBH is much higher in horses born in Iceland and exported to the continent, compared to Icelandic horses born in a *Culicoides*-rich environment. The specific allergens causing IBH have been identified and expressed as recombinant proteins in *E. coli*. The aim was to express allergens in barley grain to use in oral immunotherapy against IBH.

Methods: Cul n 2 was expressed in barley grain with the Orfeus technique and in insect cells with the Bac-to-Bac system. Cul n 2 transgenic barley flour of 50 g was mixed in NaCl with hydroxyethylcellulose and two horses treated six times orally with total 350 g of barley containing 35 mg rCul n 2 over a period of 4 months. Two control horses were treated with unmodified barley. The horses received the barley mixture in special hollow bits. The mixture was slowly consumed in 4–5 h. Serum and saliva samples were collected before and 2 weeks after each treatment for the analysis of antibody responses in Western blot (WB) and ELISA.

Results: The allergen Cul n 2, a hyaluronidase originating from *C. nubeculosus*, was expressed in barley grain. When tested in WB on rCul n 2 purified from insect cells, specific IgG response could be detected in serum from the horses treated with the transgenic barley. IgG subclasses were tested in ELISA on rCul n 2 produced in *E. coli*. The response was mainly of IgG4/7 subclass and to some extent IgG1. No detectable IgG5 or IgG6 was induced and no IgE.

Conclusion: Specific antibody response could be obtained in horses using oral treatment with transgenic barley flour expressing an allergen. Further studies will show whether this approach is a useful alternative for the prevention and treatment of equine IBH.

WS5.4

Vaccination of Horses Against Insect Bite Hypersensitivity: Intralymphatic Injection with Allergens in Alum or Alum and Monophosphoryl Lipid A

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Background: Insect bite hypersensitivity (IBH) is an IgE-mediated allergic dermatitis of horses caused by bites of insects of the genus *Culicoides* spp. (midges) that do not exist in Iceland. All breeds of horses can be affected but horses born in Iceland and exported to the continent are more frequently affected than Icelandic horses born abroad. We have isolated the respective *Culicoides* allergen genes and expressed the proteins in *E. coli*.

Objectives: The objective was to compare two different adjuvants for intralymphatic vaccinations using four purified recombinant allergens with the final aim to develop immunotherapy against IBH.

Methods: The allergens, rCul n 3, 4, 8 and 10, were produced in *E. coli* and purified. Twelve healthy Icelandic horses were vaccinated intralymphatically three times with four recombinant allergens (10 μg/vaccination), six with the allergens in alum and six with the allergens in alum and monophosphoryl lipid A (MPL). Serum was collected for the analysis of antibody response and the horses were tested for allergy.

Results: Immunization with allergens in both alum and alum/MPL gave strong specific IgG responses. The IgG subclass antibodies induced against the four allergens were mainly IgG1 and IgG4/7 and to some extent IgG5. There was no detectable induction of IgE nor any indication of IgE-mediated reaction as the horses did not respond to the recombinant allergens in skin test and *Culicoides* extract stimulation in a sulfidoleukotriene release test was negative.

Conclusions: Intralymphatic vaccination with small amount of purified recombinant allergens in adjuvant induced strong specific IgG response and not IgE. The difference between alum and alum/MPL was not significant but further experiments with analysis of the cytokine profile and more horses are needed, as the results look promising for the development of immunotherapy against IBH.

WS5.5

Culicoides Nubeculosus Hyaluronidase an Allergen (Cul n 2) in Insect Bite Hypersensitivity of Horses: Expression in Insect Cells and Barley Grain and Generation of Cul n 2 Specific Monoclonal Antibodies

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Background: Hyaluronidase from biting midges, *Culicoides* spp, is one of the allergens causing insect bite hypersensitivity (IBH), an IgE-mediated dermatitis of horses. *Culicoides* spp. are not indigenous to Iceland and the prevalence of IBH is very high in horses born in Iceland and exported to the continent. The hyaluronidase from *C. nubeculosus* has been isolated and expressed in *E. coli*, its allergenicity established and the allergen termed Cul n 2. The objective was to express in insect cells and in barley the most important allergens causing IBH with the final aim of developing an immunotherapy against the IBH.
Methods: Monoclonal antibodies (mAb) were generated against *E. coli*-produced Cul n 2 using the hybridoma technique. rCul n 2 was expressed in insect cells with the Bac-to-Bac system (Invitrogen). rCul n 2 was expressed in barley grain with the Orfeus technique [Magnusdottir A., et al. (2013) Trends Biotechnol. 31:572–80]. Protein detection was carried out with SDS-PAGE electrophoresis, Coomassie staining and Western blot.
Results: The allergen Cul n 2 was expressed in insect cells using baculovirus. Cul n 2 was also expressed in barley grain. Third-generation seeds contain 1 mg rCul n 2 per 10 g grain and are available in a seed bank for future production. The Cul n 2-specific mAb binds to the *E. coli* expressed Cul n 2 as well as rBac-Cul n 2 and barley produced rCul n 2. IgG from horses vaccinated with *E. coli*-produced rCul n 2 in adjuvant bound to both rBac-Cul n 2 and rCul n 2 from barley.
Conclusion: It is of importance to express, in insect cells, the allergens that cause IBH for monitoring the immune response following immunotherapy. Barley seeds are an excellent source of recombinant proteins, ideal for their stable storage.

WS5.6

Lower IL-21 Levels at Birth are Associated with Parental Allergic Heredity

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Children are born with an immature immune system that develops during their first years of life under the influence of environmental factors. In some individuals, non-hazardous antigens induce IgE production and cause allergic disease. The prevalence of allergy has increased during the last decades. A reduced exposure to microbial products is thought to be a contributing factor. In an attempt to better understand how an immune response could relate to allergy development during childhood, we studied the cytokine profiles of children from a well-characterized allergy cohort comprising a group of 281 children followed from the third trimester of pregnancy until 10 years of age. Parental allergy (heredity), IgE-sensitization and manifest allergic disease have been thoroughly recorded throughout the study (up to 10 years). Plasma cytokines were measured at 0 (birth), 2, 5 and 10 years of age. Results from 105 children (37% of the cohort) showed that as expected, the children with both allergic parents presented with significantly higher levels of IL-4, IL-13 and IL-17A in their plasma at birth, when compared to children without allergy heredity. In contrast, children without allergic parents had significantly higher concentration of IL-21 in plasma at birth, when compared to children with allergic heredity. Interestingly, the IL-21 levels increased at 5 years on children with allergic heredity when compared to children with no allergic heredity. Cytokine analyses in relation to allergic outcomes will be performed. As the role of IL-21 in inhibiting IgE production has been described, our results so far indicate that IL-21 regulatory effects were diminished already at birth on children born from allergic parents. However, an increase in IL-21 levels later in life even in children with allergic heredity suggests that other factors could influence an immature immune system. Some of these factors are currently under investigation.

WS5.7

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Insect Bite Hypersensitivity of Horses: Intralymphatic Immunization of Foals with Baculovirus Vectors Expressing Culicoides Allergen

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Background: Insect bite hypersensitivity (IBH) is a recurrent seasonal allergic dermatitis of horses, a reaction to biting midges, *Culicoides* spp. The incidence of IBH is very high in horses born in Iceland and exported to the continent. The aim of the project was to develop baculoviral vectors for immunotherapy of IBH.
Methods: Two recombinant baculoviral vectors (rBac) were constructed with the allergen gene Cul n 2, a hyaluronidase from *C. nubeculosus.* Four foals were vaccinated 5× in the submandibular lymph node two with each vector and then all subsequently boosted with *E. coli*-produced Cul n 2 in monophosphoryl lipid A/alum adjuvant. Gene expression in insect cells and equine kidney cells was tested in Western blot (WB) and serological response in WB and ELISA.
Results: We constructed two baculoviral vectors, rBac-Cul n 2 with the Cul n 2 under the polyhedrin promoter and rBac-EHV-2-gB-Cul n 2, with the glycoprotein B (gB) from the equine herpesvirus 2 (EHV-2) under the polyhedrin promoter and with a mammalian expression cassette containing cytomegalovirus promoter, intron A, the Cul n 2 allergen and poly A. After inoculation of rBac-EHV-2-gB-Cul n 2, the Cul n 2 was expressed in equine cells, whereas no expression was obtained with the rBac-Cul n 2 vector. However, Cul n 2-specific antibody response was not detected in rBac-EHV-2-gB-Cul n 2-vaccinated foals but only a low response in foals vaccinated with rBac-Cul n 2. After protein boost, antibody response was similar in all foals.
Conclusions: The allergen Cul n 2 was expressed in equine cells after inoculation with the baculoviral vector rBac-EHV-2-gB-Cul n 2. However, the vector did not induce detectable antibody response in contrast to the control rBac-Cul n 2 vector. Further studies will show whether the recombinant Bac vectors can be employed in IBH immunotherapy.

WS5.8

The IL6R Expression is Downregulated by miR-451 in Tumour Progression

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IL6 was originally identified as a B cell differentiation factor. It is a multifunctional cytokine that regulates the immune response, haematopoiesis, the acute-phase response and inflammation. IL6 is produced by various types of cells and influences various cell types and has multiple biological activities through its unique receptor system. IL6 exerts its biological activities through IL6R. IL6R is involved in IL6-STAT3-HIF signalling pathway, which plays a role in cell growth and carcinogenesis. The miR-451 was found to be frequently downregulated in tumours, indicating that miR-451 could play an important role in carcinogenesis. This study uncovered the mechanism by which the miR-451 functions as a tumour suppressor. The target genes of miR-451 were determined using target gene prediction software. Then, the miR-451 mimics were introduced into RKO and Hela cells, respectively. The proliferation and invasion of cells were monitored by MTT, cell cycle and *in vitro* extracellular matrix invasion assays. Also, the angiogenesis of HUVEC cells transfected with miR-451 mimics was examined. Subsequently, IL6R, a predicted target gene of miR-451, was studied by real-time PCR, Western blotting and siRNA technologies. The mRNA and protein levels of IL6R gene were found to be downregulated in the RKO and Hela cells transfected with miR-451 mimics. Consequently, the cell proliferation was inhibited. Also, the invasion of RKO cells was suppressed. Furthermore, the angiogenesis of HUVEC cells transfected with miR-451 mimics was assayed and the decreased angiogenic ability was detected compared to the controls. All these results were validated by IL6R siRNA experiments. The IL6R gene is a target gene of miR-451. The miR-451 behaves as a tumour suppressor, probably by targeting the IL6R pathway.

WS5.9

NK Cells and ALL: A Rat Model of T Cell Lymphoblastic Leukaemia

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Natural killer (NK) cells play a central role in tumour immunosurveillance and exert both direct and indirect effects by killing transformed target cells and by producing cytokines to regulate the immune system. Despite this, many cancer cells escape NK immunosurveillance and appear to be resistant to NK-mediated cytotoxicity. Several studies have demonstrated that acute lymphoblastic leukaemia (ALL), and in particular T cell-derived leukaemias, are among these.

We have established a rat model of ALL, utilizing a primary T cell leukaemia isolated from PVG rats (Roser; Roser *et al.*, 1975). To study the effect of the leukaemia on the functionality of NK cells, we have combined *in vitro* assays and *in vivo* experiments to investigate changes in the phenotype and in the functionality of NK cells in the context of ALL.

We tested *in vitro* the ability of rat NK cells to form conjugate, to kill and/or to produce cytokines in response to Roser leukaemia cells. We found that NK cells form few conjugates with Roser leukaemia cells, and poorly killed the leukaemic blasts in a 4-h ^{51}Cr release assay. However, addition of IL-2 to the co-cultures of NK cells and Roser leukaemia cells improved the killing activity towards the Roser leukaemia cells. The production of IFN-γ by NK cells was not affected by the presence of Roser leukaemia cells in the culture. We also investigated changes in NK phenotype upon exposure to Roser leukaemia cells, but found no difference in the NK phenotype after co-culture. In order to define the mechanism of reduced NK functionality, Roser leukaemia cells have been tested for the expression of known ligands for the NK activating receptor NKG2D and the supernatant of NK-Roser leukaemia cells co-culture has been screened for the presence of soluble NKG2D ligands and other known immunosuppressive factors.

WS5.10

Disease Relapse After Tyrosine Kinase Inhibitor Treatment Discontinuation in Chronic Myeloid Leukaemia is Related to Both Low Number and Impaired Function of NK Cells

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Chronic myeloid leukaemia (CML) is caused by a translocation in the haematopoietic stem cell resulting in BCR-ABL1 oncokinase. The inhibition of BCR-ABL1 with tyrosine kinase inhibitors (TKIs) has significantly improved the prognosis of CML.

Recent reports suggest that approximately 40% of CML patients who have achieved optimal therapy response (sustained complete molecular remission) are able to stop TKI treatment without disease relapse. However, there are no predictive markers for successful therapy discontinuation. Therefore, we set up an immunological substudy in the ongoing pan-European EURO-SKI stopping study. We aimed to identify predictive biomarkers for relapse/non-relapse after TKI discontinuation, to understand more on the mechanisms of immune surveillance and to study the effects of TKI treatment on the immune system.

Basic lymphocyte analysis was performed at the study entry and at 1, 6 and 12 months after the TKI discontinuation (currently 94 patients included in the substudy). In 43 patients, more detailed immunophenotypic and functional analyses were performed at the same time points.

Interestingly, patients who were able to maintain remission had increased NK cell counts already before stopping the

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treatment compared to patients who relapsed. Furthermore, the phenotype of NK cells was more cytotoxic (CD57+CD16+CD62L-), and also their function (degranulation and direct cytotoxicity) was better in patients who did not relapse. No differences were observed in the function or the numbers of T cells at the time of treatment discontinuation. However, the Th1 type of cytokine secretion by CD8+ T cells enhanced after the treatment discontinuation in patients who were able stay in remission.

In conclusion, the NK cell number and their function may predict disease relapse after TKI discontinuation. This may have impact on the future stopping trials. In addition, it further illustrates the importance of the immune system in the successful long-term treatment of CML.

WS5.11

Chronic Lymphocytic Leukaemia Cells with Trisomy 12 Home to the Bone Marrow in a CXCR4-Independent Manner

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Trisomy 12 (tri12) defines a subgroup among CLL patients with specific clinical features, which is associated with increased expression of the negative prognostic marker CD49d, the α-subunit of the VLA-4 integrin. VLA-4 is crucially involved in homing of CLL cells to bone marrow (BM). This process is thought to depend on inside-out activation of VLA-4 upon CXCL12–CXCR4 interaction augmenting the arrest of the cells on the VCAM-1 presenting endothelium. The interplay between VLA-4 and CXCR4 in tri12 CLL was investigated by a combination of cytometrical, videomicroscopical and *in vivo* assays. High expression of CD49d was accompanied by lower levels of CXCR4 in tri12 CLL. Short-term adoptive transfers of CLL cells to immune-deficient mice were performed to analyse their homing capacity. BM homing depended on functional VLA-4 expression. However, while this homing could be diminished by inhibition of the CXCL12-CXCR4 axis in non-tri12 CLL cells, tri12 CLL cells homed independently of these signals. Migration assays under shear flow were used to test whether VLA-4 could undergo CXCL12-induced activation and support the adhesion of CLL cells to a VCAM-1 substrate. While CXCL12 enhanced arrests of non-tri12 CLL cells, tri12 CLL cells did not rely on this chemokine. Analysing VLA-4 conformations upon chemokine stimulation in real-time, CXCL12 was found to fail to induce inside-out activation of VLA-4 in tri12 CLL. Our results suggest that tri12 CLL use a different pathway for integrin activation and provide a mechanistical basis explaining, at least in part, the peculiar clinical features of tri12 CLL.

WS5.12

The Spiegelmer® NOX-A12 Mobilizes CLL Cells from Lymphoid Organs and Prevents Their Recirculation

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NOX-A12 is a novel Spiegelmer®-based antagonist of CXCL12, a stromally expressed chemokine involved in chronic lymphocytic leukaemia (CLL) cell trafficking to and retention within bone marrow (BM) and other lymphoid organs.

Here, the *in vivo* efficacy of NOX-A12 to mobilize CLL cells from their protective lymphoid microenvironment to the peripheral blood, thereby potentially sensitizing them to cytotoxic agents, and to prevent their recirculation, was investigated.

Using the Tcl1 transgenic transplantation model that mimics human CLL, NOX-A12 demonstrated even at low doses (10 mg/kg) a considerable potential to mobilize CLL cells and other lymphocytes as relative CLL cell and absolute lymphocyte blood counts raised within 1 h up to 200% and reached a peak after 6 h. Higher doses lead to a prolonged mobilization duration. Short-term adoptive transfers of human CLL cells into immune-deficient mice revealed a reductive effect of NOX-A12 treatment on CLL cell recirculation to BM in the majority of clinical CLL subgroups. Next, CXCR4 surface levels of CLL cells before injection and after cell had homed to BM were determined. Homed CLL cells expressed increased CXCR4 surface levels and CXCR4 levels were further increased upon CXCL12/CXCR4 inhibition with NOX-A12.

Summarizing, our data demonstrate a high efficiency of NOX-A12 to mobilize CLL cells and to prevent their recirculation into protective lymphatic organs. Abrogating CXCL12/CXCR4 signalling in combination with chemotherapy may provide an attractive approach for the

treatment of CLL, which is currently evaluated using the Tcl1 transgenic transplantation model.

WS5.13

Cell Surface Transport Undercover: The Expression of the Soluble and Surface-Bound NKG2D-Ligand ULBP2 is Dependent on Endosomal Regulation

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Soluble ULBP2 is a marker for poor prognosis in several types of cancer. In the present study, we demonstrate that both soluble and cell surface-bound ULBP2 is transported via a so far unrecognized endosomal pathway. ULBP2 surface expression, but not MICA/B, could specifically be targeted and retained by affecting endosomal/lysosomal integrity and PKC activity. This novel pathway was identified through screening experiments by which methylselenic acid (MSA) was found to possess remarkable NKG2D-ligand regulatory properties: MSA induced MICA/B surface expression but on the contrary, MSA dominantly blocked endosomal transport of ULBP2 through a sustained PKC inhibitory activity. Notably, by targeting this novel pathway, we could specifically block the production of soluble ULBP2 from different, primary melanomas. Our findings strongly suggest that the endosomal transport pathway constitutes a novel therapeutic target for ULBP2-producing tumours.

WS5.14

The Role of NK Cells in T Cell Acute Lymphocytic Leukaemia

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NK cells have an important role in protecting the body from cancer development. However, in cancer patients, NK cells have demonstrated decreased function. The purpose of this study was to get a better understanding on how NK cell function changes during T cell ALL progression in the rat. To test this PVG, RT7b rats were injected with Roser leukaemia and sacrificed at various time points. Flow cytometry was utilized to measure differences in NK cell subsets, maturation level, activation markers and functional ability of NK cells with ALL compared to healthy controls. We have previously characterized a NKR-P1B^{bright} subset found in the blood as terminally differentiated effector NK cells. We found that the percentage of NKR-P1B^{bright} cells in blood decreased around day 14, which is the same time point in which low levels of leukaemia cells can be detected. At the same time, there is an increase in CD11b+CD27- mature cells. These results suggest that the NK cells are affected by the leukaemia cells by day 14 with an increase in mature cells and most likely the activation and subsequent death of the NKR-P1B^{bright} subset. A decrease in NK cell function was measured after day 20. By this time point, the ALL cells were the most numerous cells in the blood. This suggests that NK cell function decreases as the leukaemic burden increases.

WS5.15

Surface-Mediated Priming During In Vitro Dendritic Cell Generation

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Introduction: *Ex vivo*-generated dendritic cells (DC) are most commonly generated from monocytes by the addition of IL-4 and GM-CSF using standard cell culture plastic dishes. However, the effects of the plastic surface on the developing DC have been poorly investigated so far. In this work, we have compared a standard plastic dish with four different, mainly non-adhesive, surfaces in order to elucidate the effect of the surface during the differentiation process.

Methods: The five surfaces were either purchased or prepared locally. Monocytes were isolated using the Monocytes Isolation Kit II from Miltenyi Biotec and cultured for 3 days in the presence of IL-4 and GM-CSF. Time-lapse videos were recorded on three time points (one each day), and the phenotype of the cells was analysed by flow cytometry. The cytokine profiles were analysed in cell-free supernatants using a 25-plex Luminex assay.

Results: Using non-adhesive surfaces did not alter the production of most cytokines significantly. However, the use of non-adhesive surfaces led to a significant reduction in expression of CD14 and CD38 and a significant increase in expression of CD86 compared to standard culture dishes. Interestingly, the expression levels of DC-SIGN and PD-L2 were also reduced significantly on cells cultured on non-adhesive surfaces.

Conclusion: Although cytokine levels are only slightly affected, standard cell culture dishes give rise to differences

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in the phenotype of monocyte-derived dendritic cells compared to non-adhesive surfaces. The surface-mediated priming should therefore be considered when aiming to induce specific immune responses.

WS5.16

Method Development for the Identification of HLA-DR-Bound Peptides

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Introduction: The identification of HLA-DR-bound peptides present a valuable tool to identify disease-specific antigen(s) in chronic inflammatory and autoimmune disorders. Our group has previously published a method, where HLA-DR-bound peptides from the deep airways could be identified from 800 million bronchoalveolar lavage (BAL) cells pooled from 16 patients. Because BAL from healthy non-smoking individuals typically only contains 10–15 $\times$ 10^6 cells, our aim was to increase the sensitivity in order to investigate individual subjects.

Methods: Cells were collected by bronchoalveolar lavage. Crude membranes were isolated using differential centrifugation and solubilized using non-ionic detergents. HLA–peptide complexes were immunoaffinity-purified and the peptides eluted using acidic conditions. Peptides were analysed using on-line nanoLC-MS/MS (Q-Exactive), followed by identification by searching the Human IPI database using the Mascot search engine.

Results: The enrichment for HLA-DR complexes prior to immunopurification reduced the amount of non-specific peptides identified in the samples. As expected, the number of peptides and proteins identified was highly dependent on the number of cells used for the purification. While we identified 606 peptides from a sample with 85 $\times$ 10^6 cells, we only found 44 peptides from 10 $\times$ 10^6 cells. The majority of identified peptides matched specific regions on the protein sequences and formed overlapping clusters, which potentially correspond to the HLA binding pockets. The peptide lengths, with a median of 15 amino acids, were consistent with literature. Comparing samples from different patients showed differences in both protein and peptide level. Some of these differences could be correlated with the HLA type of the patient.

Conclusion: The improved method will enable us to compare peptide repertoires of BAL cells between patients and healthy individuals. Further investigations will be performed to validate the identified peptides regarding disease specificity and binding prediction. Such peptides may trigger pathogenic T cell responses and could become tools for antigen-specific immunotherapy.

Workshop 6. Infectious Diseases and Vaccination/Immune Intervention and Therapy

WS6.1

A Single Point Mutation in the spxB Gene of Streptococcus pneumoniae Dramatically Alters the Interaction with Macrophages and Increases the Severity of Infection

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Streptococcus pneumoniae is a common colonizer of the human nasopharynx, especially in preschool children. At least 93 different serotypes have been identified, of which some such as serotype 1 are associated with a high invasive disease potential. An important virulence factor for *S. pneumoniae* is pyruvate oxidase, which is responsible for the production of hydrogen peroxide and is encoded by the *spx*B gene. Hydrogen peroxide allows *S. pneumoniae* to outcompete other bacterial species such as *Haemophilus influenzae* and *Neisseria meningitidis* during colonization of the nasopharynx.

Mutations which disrupt the expression of the *spx*B gene alter the interaction between *S. pneumoniae* and host cells. *In vitro* data for both human and murine macrophage cell lines showed a reduction in the binding of *S. pneumoniae* strains with impaired *spx*B function compared to strains with the wild-type gene. There was also a reduction in the subsequent phagocytosis of the *spx*B mutant strains. *In vivo* experiments support this finding that in an intraperitoneal infection model, C57BL/6 mice infected with a strain with impaired *spx*B function had a shorter survival time than mice infected with the wild-type strain. Furthermore, mice infected with the mutant also had higher bacterial titres at the time of sacrifice. To mimic systemic disease, mice were infected intravenously with wild-type and spxB mutants of serotypes 1 and 4. For both strains, bacterial titres were consistently higher throughout the infection for the spxB mutants than for the wild-type strains. Interestingly, for serotype 1 but not for serotype 4, this translated into a shorter median survival time. How the interaction between the innate immune system and *S. pneumoniae* changes due to mutations in the spxB gene still remains unsolved. It is likely that the altered interaction is due to indirect effects of an impaired *spx*B gene and not directly attributable to hydrogen peroxide.

WS6.2

Obesity and Risk of Infection

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Background: Obesity is associated with increased risk of a range of diseases. However, the association of obesity with risk of infection among otherwise healthy individuals has not been established.

Methods: The project included 37,634 participants in The Danish Blood Donor Study (DBDS), which was initiated 1 March 2010. All participants completed a standard questionnaire on smoking status, alcohol consumption, physical activity, diet and various body measurements, etc. Obesity was defined according to body mass index (BMI) using WHO definitions: BMI < 30 kg/m^2 (reference) and BMI ≥ 30 kg/m^2 (obese).

Infections among participants were defined as relevant ICD-10 codes of infections in the Danish National Patient Registry and as relevant ATC-codes of antibiotics in the Danish Prescription Registry.

To measure risk of infection, multivariable Cox proportional hazards analyses with BMI, waist circumference, prescription of antibiotics, age, current smoking status and sex as predictors were performed with risk time defined as time since inclusion.

Results: The participants provided a total of 105,833 risk years and 1156 participants were admitted to the hospital due to infection, while 15,723 received prescriptions of antibiotics during 58,259 risk years.

Obesity was associated with an increased risk of all-cause infection for both men (hazard ratio (HR) = 1.50, $P < 0.0001$, 95% confidence interval (CI): 1.24–1.74) and women (HR = 1.44, $P = 0.004$, CI: 1.12–1.84). The effect of obesity was similar to that of current smoking (men: HR = 1.26, $P = 0.023$, CI: 1.03–1.50; women: HR = 1.37, $P = 0.003$, CI: 1.11–1.68). Similarly, obesity was strongly associated with prescription of antibiotics (men: HR = 1.21, $P < 0.0001$, CI: 1.13–1.31; women: HR = 1.16, $P < 0.0001$, CI: 1.09–1.24).

For specific infections, obesity was most strongly associated with risk of infection of the skin and subcutaneous tissue, cystitis among women and abscesses.

Conclusions: In a large cohort of healthy individuals, obesity was statistically significantly associated with an

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increased risk of infection, similar to the risk associated with smoking.

WS6.3

The Danish Blood Donor Study: Nasal and Throat Colonization with Staphylococcus aureus Among Healthy Danish Blood Donors

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Introduction: *Staphylococcus aureus* is a commensal but it is also a potent pathogen that can cause a wide variety of infections. Approximately 25–30% of the healthy adult population are carriers of *S. aureus*. Colonization is a complex interaction between the bacteria and the host immune system and it is still incompletely understood. The aim was to describe the prevalence of nasal and throat colonization with *S. aureus* among healthy adults and identify immunological predictors of colonization.
Methods: Our study will comprise 5000 participants from the Danish Blood Donor Study. So far, 215 participants have been swabbed from the nose and throat. Samples were cultured on selective *S. aureus* ID chromogenic agar. To enhance sensitivity, all samples negative for *S. aureus* in the primary culture were subcultured from enrichment broth. Identification of *S. aureus* was based on the selective chromogenic agar and confirmed with MALDI-TOF (matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer). Plasma and DNA is available from all participants.
Results: Preliminary results reveal a prevalence of 54% carriers of *S. aureus*. The colonization rates are follows: 28% colonized in the anterior nares and throat concomitantly, 13% only colonized in anterior nares and 13% only colonized in the throat. By using the enrichment broth, the detection of *S. aureus* in the anterior nares and throat was increased by 8% and 14%, respectively.
Summary: The prevalence of *S. aureus* colonization was comparable with previous reports. Our results indicate that the anterior nares and throat are equally colonized with *S. aureus* in contrast to the report from recent studies. The results also emphasize the importance of using an enrichment broth in the detection of *S. aureus*. The participants will be genotyped for relevant single nucleotide polymorphisms to identify predictors of colonization.

WS6.4

Mycobacterium marinum Virulence Factors: Tuberculosis by Host Immunomodulation

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Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. This reemerging disease is carried by one-third of the world's population, and its medical treatment is losing its grip, making the disease one of the most prominent threats of the human kind. For these reasons, the World Health Organization gives the highest priority in fundamental research to better understanding of the host–pathogen interaction. For this, a natural TB model system of zebrafish and *Mycobacterium marinum* is highly applicable. Even though much of TB's infection biology remains to be understood, the pathogen is known to be able to persist in the host by evading and exploiting the host immune system. Our recent results suggest that different infection profiles are reflected by different adaptive immune response profiles: a well-controlled infection is associated with an induced humoral Th2 response (type 2 helper cell), whereas its absence correlates with progressive disease. In this project, we will use a *M. marinum* mutant library generated recently by Dr. Astrid van Der Sar's research group (VU University Medical Center, Amsterdam, Netherlands). By initial screening of virulence factor mutants and by subsequent scrutinizing of mutants causing altered balance of host Th response, the project aims to characterize mycobacterial virulence factors acting through host immunomodulation. Such factors could be consistent drug targets by letting the adaptive immune system from mycobacteria's leash.

WS6.5

Immunomodulation of Latent Tuberculosis in the Mycobacterial Infection Model of Adult Zebrafish

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Tuberculosis is one of the deadliest bacterial diseases worldwide. According to World Health Organization, there were 8.6 million tuberculosis patient and altogether 1.3 tuberculosis-related deaths in 2012. Furthermore, it has been estimated that up to 90% of the infected has

latent asymptomatic infection that can reactivate to infective disease. Active tuberculosis is difficult to cure and at the moment the standard antibiotic regimen lasts for minimum of 6 months. Latent tuberculosis is even harder to treat as there is an increased population of dormant mycobacteria that replicate rarely and have thickened cell wall.

Our research group has developed mycobacterial infection model of adult zebrafish in which latent disease can be spontaneously developed without the help of chemotherapeutics. In this infection model, it is possible to test new treatments for latent tuberculosis either by *intraperitoneal* injection of drugs or by feeding fish *per os.* 'Wake and whack' is one approach for treating latent tuberculosis, where dormant mycobacteria are first sensitized with immunosuppressants and then these newly reactivated mycobacteria are killed with antibiotics. In this experiment, we will use immunosuppressants such as dexamethasone and rolipram as reactivators of dormant mycobacteria and common antituberculosis antibiotics such as isoniazid and ethambutol. Our preliminary data indicate that adult zebrafish is a promising model for preclinical testing of latent tuberculosis treatments and that combining delicate immunosuppression and effective antibiotic treatment can improve the treatment outcome.

WS6.6

Tuberculosis Vaccine Antigen Screen in the Adult Zebrafish Model

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Tuberculosis remains a major global threat and an improved vaccine to replace the current bacillus Calmette–Guérin (BCG) vaccine is needed. Moreover, methods for boosting the immunity of people already vaccinated with BCG are lacking. As a small, non-mammalian vertebrate with a fully developed immune system, the zebrafish (*Danio rerio*) is a new, attractive and affordable model for vaccine research. Zebrafish can be vaccinated against mycobacteriosis and experimental infection with *Mycobacterium marinum* – a fish pathogen closely related to *Mycobacterium tuberculosis* causing human tuberculosis – can be used to evaluate the efficacy of vaccine candidates. This project uses an adult zebrafish model for screening through mycobacterial antigens shared by *M. tuberculosis* and *M. marinum* for their protective effects against mycobacteriosis. The screening is performed using DNA-based vaccination that represents an alternative for immunizing against tuberculosis and a tool for vaccine antigen screening. Antigens associated with different bacterial metabolic states are included in the screen to specifically target different infection phases. Our aim is to identify antigens or combinations of antigens that efficiently induce protection against mycobacteriosis. These antigens could potentially be used as DNA- or peptide-based vaccines themselves, or be used as a booster vaccine for BCG. Our long-term goal is to find safe and effective ways to prevent different forms of tuberculosis.

WS6.7

Correlation of Viral Load with Fibroindex Stage of Chronic Hepatitis C Patients

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Introduction: Fibroindex was first described by Koda et al. (1) as a simple, non-invasive indirect marker of liver fibrosis; it was derived from the platelet count, AST, and gammaglobulin measurements. Aim of the study: The aim of the present study was to evaluate the fibrogenic marker: fibroindex as a non-invasive marker of liver fibrosis in chronic hepatitis C and to correlate its serum levels with the stage of fibrosis assessed by histopathological staging of liver biopsy and virological characteristics including HCV RNA titres.

Patient and Methods: This study was carried out on 40 patients with evidence of chronic hepatitis C (positive HCV-Ab and positive HCV RNA by PCR), and they were (35) male and (5) female. All cases were selected from outpatient clinic of the hepatology unit of research institute for tropical medicine (2011). The patients had liver fibrosis from F1 to F4. Patients with no advanced liver fibrosis (F1–F2) were considered as control. Fibroindex was calculated according to the equation described by related authors. (fibroindex = 1.738 − 0.064 (platelets [$\times 104/mm^3$]) + 0.005 (AST [IU/l]) + 0.463 (gamma globulin [g/dl])).

Results: The study revealed no significant correlation between PCR with both liver biopsy and the fibroindex. Also, there was no significant correlation between fibroindex and liver biopsy. We obtained two cut-off values (0.88) and (0.84) and compared these two cut-off values with the respective original report in which they set two cut-off values in order to determine whether significant fibrosis was present ($\geq$2.25) or not ($\leq$1.25); we were not able to differentiate patients with cirrhosis (F4) or significant fibrosis (F2, F3) from those without significant fibrosis (F1).

Conclusion: Fibroindex had low specificity and positive predictive value in identifying patients with significant or severe fibrosis. In addition, its sensitivity was limited and not sufficient too.

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WS6.8

Polysaccharide-Induced Hyporesponsiveness to Pneumococcal Conjugate Vaccines Depends on the Dose and Type of the Polysaccharide

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Introduction: Pneumococcal polysaccharide (PPS) can decrease antibody (Ab) response to a subsequent immunization with a pneumococcal conjugate vaccine (PCV). This effect is evident from both experimental and clinical studies. We assessed whether PPS-induced hyporesponsiveness was dependent on PPS dose and serotype.
Methods: Neonatal mice were primed with ten-valent PCV (PCV10), saline or 23-valent PPS (PPV23) at three different doses and boosted 16 days later with PCV10. PPS-specific Abs in serum were measured and PPS-specific antibody-secreting cells (AbSC) enumerated in spleen and bone marrow 6 weeks after the first immunization.
Results: PPV23 priming decreased the Ab response to subsequent PCV10 immunization for serotypes 1, 4, 5, 9V and 18C, but had no effect on responses to serotypes 7F and 14. The hyporesponsiveness was dependent on the PPV23 dose, and the lowest dose did not affect the response to 9V and 18C. Priming with PPV23 also reduced the number of AbSCs specific for three of four serotypes tested (1, 4 and 18C) in spleen and bone marrow.
Conclusion: PPV23 priming induced dose-dependent hyporesponsiveness to PCV10, reduced AbSCs in spleen and BM, resulting in long-lasting reduction in serum IgG Abs specific for the majority of serotypes. The effect was dependent on the type of polysaccharide; specifically, PPS with a neutral charge had no effect.

WS6.9

Vitamin D3 Administration to MS Patients Leads to Increased Serum Levels of TGF-Beta

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Background: Vitamin D deficiency has been identified as an environmental risk factor for multiple sclerosis and low serum levels of vitamin D correlate with increased risk of MS relapses and disease progression. In healthy volunteers, vitamin D supplementation (5000–10,000 IU/day) has been associated with an increase in IL-10 production by PBMC and reduced frequency of Th17 cells (Aideen et al. 2012). There are only few studies of effect of vitamin D supplementation on immune responses in patients with MS. Aims of the study: In this study, we investigated the effect of weekly vitamin D or *placebo* supplementation on the serum levels of LAP (latency-associated peptide of TGF-β), IFN-γ, IL-17A, IL-10, IL-2, IL-22, IL-9, IL-13, IL-6, IL-4, IL-5, IL-1β and TNF-α.
Materials and Methods: This study was conducted on a cohort of patients who participated in The Finnish Vitamin D Study (Cholecalciferol as Add-on Treatment to Subcutaneously Administered Interferon beta 1-b for Treatment of MS), a double-blind, randomized, parallel group 1-year multicentre trial (Soilu-Hänninen et al. JNNP 2012). In this trial, patients were randomized either to cholecalciferol 20,000 IU/week of *placebo* capsules for 12 months. Serum samples were obtained at screening and at 1, 2, 3, 6, 9 and 12 months. For this study, we used samples taken at screening and at 12 months. Concentrations of LAP (TGF-β), IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, TNF-α and IL-1β were determined using commercial fluorescent bead immunoassay kits (Human Th1/Th2/Th9/Th17/Th22 13plex Kit, and Human LAP Simplex Kit, eBioscience, USA).
Results: LAP levels increased significantly in the vitamin D-treated group from a mean of 47 (S.E. 11) to 55 (S.E. 14) nM in 12 months. *Placebo* treatment had no significant effect on LAP levels. The levels of IFN-γ, IL-17A, IL-2, IL-10, IL-9, IL-22, IL-6, IL-13, IL-4, IL-5, IL-1β and TNF-α did not change statistically significantly in either group.
Conclusions: The effect of vitamin D in MS may be mediated by increased TGF-β levels.

WS6.10

Development of Anti-Idiotypic Antibodies Towards the Beta Cell-Specific IC2 Antibody

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A major objective in diabetes research is to identify factors that can prevent beta cell death. An attempt of using anti-idiotypic antibody-mediated immunotherapy for the prevention of type 1 diabetes has been performed in the NOD mice model of the disease. Anti-idiotypic antibodies towards GAD65 antibodies were shown to significantly delay the onset and reduce the incidence rate of diabetes in the mice model. Increased levels of GAD65 autoantibodies

are well known in the development of type 1 diabetes, but the lack of beta cell specificity of GAD65 anti-idiotypic antibodies means that the search for beta cell-specific immunotherapy in type 1 diabetes continues. NKT cells have been shown to be important in the regulatory mechanisms preventing diabetes *in vivo* and the beta cell-specific monoclonal rat antibody IC2 has been shown to inhibit the cytokine (IL-2) secretion of these cells *in vitro*. Therefore, anti-idiotypic antibodies towards IC2 might have higher therapeutic potential in the prevention or cure of type 1 diabetes compared to the anti-idiotypic antibodies against anti-GAD65.

We have successfully developed syngenic IC2-specific anti-idiotypic antibodies through rat–rat hybridoma fusions. Initially, immunizations of BB/BBDR+/+ rats with IC2 were performed, to develop an ELISA assay for the detection of the IC2-specific anti-idiotypic antibodies as well as to define the immunogenicity of IC2. A second round of immunizations was used for the hybridoma fusions followed by limiting dilution cloning for propagation of the hybridoma cultures towards monoclonality. Large-scale production of the antibodies was carried out in CELLine tanks, and purification was performed through immunoaffinity chromatography. Finally, characterization of ten of the anti-idiotypic antibodies has been initiated with the intention of choosing which to use for *in vivo* testing in diabetes prevention of the diabetes-prone BB rat. Further characterization will have to be done, but our data entail promising results in regard of the *in vivo* potential of the IC2-specific anti-idiotypic antibodies.

WS6.11

Development of Antidrug Antibodies in Multiple Sclerosis Cohorts in Europe – Overview of Interferon Beta and Natalizumab Cases Tested for ADA in Different Countries

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A joint effort from academia and industry resulted in 2012 in a collaboration called ABIRISK (Anti-Biopharmaceutical Immunization: prediction and analysis of clinical relevance to minimize the RISK) that was funded by IMI (Innovatives Medicines Initiative) and EFPIA (European Federation of Pharmaceutical Industries and Associations). One of the tasks of this collaboration was to set up and initiate the use of a common database platform (TranSMART) and to start loading data from all laboratories within this collaboration that analyse antidrug antibodies (ADAs) against biopharmaceuticals used in either multiple sclerosis (MS), rheumatoid arthritis, inflammatory bowel disease or haemophilia A.

The aim was to set up the TranSMART platform for querying, analysing and storing retrospective data within ABIRISK. This project is well underway and the first retrospective data cohorts have been loaded. The task of curating data and uploading it is in progress and MS cohorts with retrospective data from five countries (Sweden, Austria, Switzerland, Germany and Denmark) have been loaded, are on the way to be loaded or are nearly finished preparing data for upload into TranSMART. Altogether, these uploads contain over 12,000 patients with over 33,000 test results from serum samples and their information can be queried for information regarding ADAs for future analyses.

Questions like 'how many patients in each country have been tested for ADA?' will be compared with regard to population size, treatment use and expected MS population size. Difference in ADA positivity between countries and if this correlates with age and gender will be determined. Healthcare-related issues like the use of different treatment preparations, ADA analysis methods and sampling routines will be analysed. Early results from these analyses will be presented.

Work Package 4 within ABIRISK would like to acknowledge the contribution of IDBS (ID Business Solutions) to the curation and hosting of ABIRISK data.

WS6.12

Neither Interferon Beta Nor Natalizumab Treatment Influences Serum Anti-HHV-6 IgG Levels

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Background: Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system. It is a complex disease where both genetic and environmental factors contribute to disease susceptibility. Human herpesvirus 6 (HHV-6) has been associated with MS disease activity. The first-line treatment of MS is interferon beta (IFN-β), but the exact mechanism by which its beneficial effect is achieved is not clear. Another treatment is natalizumab, a monoclonal antibody directed against

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VLA4 that blocks CD4+ T cells from entering into the CNS. Previous studies have observed that 1 year of IFN-β treatment can reduce HHV-6 prevalence in PBMC and that natalizumab-treated MS patients have higher levels of anti-HHV-6 serum IgG compared to untreated MS patients, suggesting that both treatments might influence immune surveillance of HHV-6.

Aim: In order to further investigate the role of two commonly used MS treatments on a virus associated with MS disease, we studied the effect of IFN-β and natalizumab on antibody levels against HHV-6.

Material and Method: Serum IgG antibody response against HHV-6 was investigated in nine treatment naïve relapsing–remitting MS patients who initiated IFNβ-1a (s.c) treatment and seven MS patients who initiated natalizumab treatment. Antibody levels were measured with ELISA before treatment start ($n_{IFN\beta} = 9$, $n_{natalizumab} = 7$) and after 6 months ($n_{IFN\beta} = 8$), 12 months ($n_{IFN\beta} = 7$, $n_{natalizumab} = 7$), 18 months ($n_{IFN\beta} = 4$) and/or 24 months ($n_{IFN\beta} = 4$) of treatment.

Results and Conclusion: Neither INFβ nor natalizumab treatment altered the anti-HHV-6 IgG levels significantly. A high interindividual variation of the anti-HHV-6 IgG levels was observed, and we stress the importance of longitudinal studies when investigating treatment effects.

WS6.13

Withdrawn

WS6.14

Combined Balneo- and NB-UVB Therapies Suppress T Lymphocytes in Psoriatic Skin Lesions

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A controlled balneo- and narrow band ultraviolet B (NB-UVB) therapy in the Blue Lagoon is a commonly used psoriasis treatment, which can suppress circulating T cells, reduce skin inflammation and improve quality of life. This study compares two treatment regimens, that is, bathing in geothermal seawater, that is, Blue Lagoon, combined with NB-UVB therapy and NB-UVB therapy alone, in suppressing epidermal T lymphocytes. Patients with psoriasis were recruited and treated for 6 weeks, five individuals for each regimen. Disease severity was recorded and skin punch biopsies were obtained from lesions on enrolment and after 6 weeks of therapy. Punches were sliced using a microtome and sections stained with antibodies specific for CD3-, CD4- and CD8-positive T lymphocytes. Numbers of lymphocytes were counted in three separate fields from each section. The combined therapy reduced CD3-, CD4- and CD8-positive epidermal T lymphocytes by 71%, 65% and 70%, respectively, and the monotherapy by 64%, 72% and 73%, respectively. No difference in the efficacy to reduce T lymphocytes was noted between the two treatment regimens. The observed reduction in epidermal T lymphocytes is in agreement with our previous data, which demonstrates reduction in circulating T lymphocytes after 6 weeks of either combined or monotherapy. Interestingly, the percentage of patients who achieved PASI 75 and PASI 90 was greater after the combined therapy (73.1% and 42.3%) than NB-UVB monotherapy (16.7% and 0%) with $P < 0.05$ in all comparisons. Taken together, the data support previous observations regarding the role of T lymphocytes in the pathogenesis of psoriasis and further strengthens the importance of epidermal T lymphocytes within the pathology and treatment of psoriasis.

WS6.15

Withdrawn

WS6.16

Health-Related Quality of Life of Individuals with Mannan-Binding Lectin Deficiency (MBLD)

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Background: Mannan-binding lectin (MBL) and ficolin-3 are initiators of the lectin pathway that is important for clearance of pathogens and apoptotic cells through complement activation. MBL deficiency (MBLD) has been associated with infectious complications but its clinical relevance in adults is unclear.

Health-related quality of life (HRQOL) of individuals with chronic illnesses has been well documented but very few have studied the impact of MBLD on HRQOL. Measuring

HRQOL has become an important foundation in clinical research to evaluate the burden of illness for individual/families and communities, to predict health outcomes and to evaluate the result and efficiency of medical and nursing interventions.

Objective: The aim of the study was to measure HRQOL of individuals with previously defined MBL deficiency.

Methods: A total of 64 individuals previously defined as MBL deficient (≤50 ng/ml) were contacted and 43 agreed to participate. The participants answered a health-related quality of life questionnaire Short Form-36 as well a detailed questionnaire focused on pulmonary and gastrointestinal infections. Also, all participants were briefly interviewed to obtain further data about their health. For comparison, 63 adult individuals were randomly selected from the general population and served as control subjects.

Results: The results demonstrate that individuals with MBLD are prone to a variety of recurrent and severe infections. Results also indicate that individuals with MBLD experience depression, anxiety and social isolation. Repeated or constant pain and fatigue was also common among the individuals.

Conclusion: MBL-deficient individuals suffer from recurrent and severe forms of infections as well as a range of clinical symptoms. This suggests that the burden of MBLD in daily life may be considerable which warrants further exploration.

WS6.17

From Genetic Variations to Biomarkers

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Multiple sclerosis (MS) is one of the leading causes of neurological disabilities in Scandinavia. MS is a chronic inflammatory complex disease in which both environmental and genetic factors are involved. The aim of this project is to study the expression of MS risk genes during natalizumab treatment in serum, plasma and cerebrospinal fluid (CSF) and the clinical outcome, with the goal to identify biomarkers that can assist with treatment decisions and prognosis.

This is going to be done using two approaches, a narrow but more selective and specific approach where we are selecting to measure the expression of four soluble receptors, soluble interleukin-7 receptor alpha (sIL7Ra), soluble interleukin-6 receptor (sIL6R), soluble gp130 (sgp130) and soluble interleukin-2 receptor (sIL2R), using enzyme-linked immunosorbent assay (ELISA). We will also use a wider approach measuring the concentrations of a larger group of proteins, first 384, then potentially more, selected from the more than 15,000 expressed proteins of the Human Protein Atlas (HPA, www.proteinatlas.org) using an advanced technique called antibody suspension bead array. Selection of the analysed proteins is guided by the updated MS risk-associated genes. These two approaches for analysis will be performed in 3–4 serial serum and/or plasma samples from 90 MS patients treated with natalizumab and in paired CSF samples before and during treatment from 26 patients.

In addition, correlations between candidate MS biomarkers and clinical information and treatment outcomes will be studied. This project has the potential to provide us with biomarkers for monitoring MS patients, which will help to provide MS patients a better quality of life.

WS6.18

Intradermal Administration of Interferon Beta Induces Dermal Dendritic Cell Activation

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Background: The skin contains a dense network of dendritic cell (DC) subtypes, and it is unclear how these DCs are influenced by interferon beta (IFN-β) administration. There are indications that drug administration through the skin impact neutralizing antidrug antibody (NAb) development. We hypothesize that skin DCs are affected differently by different IFN-β preparations and that activation of these cells also correlates with individual variation determining the risk of developing NAb.

Objective: To assess the effect of IFN-β administration to a human *ex vivo* skin model on the migration and maturation of skin DCs comparing different preparations and characterizing donor variations.

Methods: Human skin was obtained from healthy subjects undergoing abdominal reduction surgery. The skin was injected intradermally with 50 μl of PBS, IFN-β-1b, or IFN-β-1a (s.c. or i.m.). A biopsy was sampled from each injection site and cultured. After 3 days, the crawl-out cells were analysed by FACS or prepared for mixed lymphocyte reaction (MLR) or T cell stimulatory assay.

Results: Intradermal injection with IFN-β-1a and IFN-β-1b enhanced the maturation status of $CD14^+$ and $CD1a^+$ dermal DC crawl-out cells as demonstrated by a significant *upregulation of activation markers CD86 and HLA-DR. The MLR demonstrated that the c*rawl-out cells from IFN-β-1a (i.m. and s.c.)-injected skin had an enhanced capacity to induce T cell proliferation compared to IFN-β-1b. Crawl-out cells from IFN-β-1a (i.m. and s.c.)-injected skin induced T helper (Th) 1 cytokine IFN gamma, whereas

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IFN-β-1b promoted the Th2 cytokine IL-4 in primed T cells.

Conclusions: Intradermal injections of IFN-β modify DC maturation and also programme DCs to promote T cell proliferation and cytokine production differently depending on preparation. This partly explains the immunologically mechanism behind the differences in NAb development depending on IFN-β preparation. Increased understanding of how the immunology of the skin impacts NAb development might lead to novel preventative regimes to minimize the risk.