Results First, we observed that up-regulation of costimulatory molecules CD86, CD80 and CD40 induced by BCR cross-linking was down-regulated after IVIg incubation and was associated with a massive reduction of tyrosine phosphorylation. Low mobilisation of intracellular Calcium, which is a hallmark of anergy, was also observed in IVIg-treated B cells. Next, we observed that following BCR stimulation, IVIg blocks BCR aggregation within lipid rafts and increases its internalisation. Consequently, BCR stimulation is not achievable and results in a low activation of the PI3K-Akt signalling pathway. Finally, we demonstrated that IVIg down-regulates NFκB activation and promotes NFAT transcription factors entry in the nucleus. These findings demonstrated that IVIg induces a selective transcriptional programme, allowing nuclear signals to be independently activated, leading to alternative B cell fates.

Conclusions Our data suggest that IVIg could induce B cells to adopt a state that results in a functional silencing, also called anergy. Our findings provide insights into the effectiveness of IVIg in treating pathologies associated with the loss of B cell tolerance. We also describe a new model to explore the complexity of positive versus negative selection in human B cells.

A5.21 **LACK OF IL-27R SIGNALING LEADS TO AN INTRINSIC B CELL DEFECT AND PROTECTION AGAINST CIA**

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Introduction The IL-27 receptor (IL-27R) is expressed on naïve T helper cells. Signaling induces Th1 differentiation and IL-10 production and inhibits Th17 differentiation. In addition, the IL-27R is also expressed on B cells, where it is linked to B cell proliferation and IgG2a class switch recombination. However, its role is not fully understood. We therefore aim to determine the role of IL-27R signalling on B cells, both in T cell independent and in T cell dependent immune responses.

Methods To determine the effect of IL-27 on B cells, we isolated naïve B cells from wild type mice and cultured them in the absence or presence of rIL-27. T cell dependent responses were investigated using the collagen induced arthritis (CIA) model in wild type and IL-27R deficient (WSX-1 deficient) mice. T cell independent B cell responses were investigated using the TNP-Ficoll immunisation model.

Results Naïve wild type B cells cultured in the presence of rIL-27 showed increased proliferation and expression of activation markers. Next, we investigated the role of IL-27R signalling in vivo in CIA. Both the incidence and severity of CIA were significantly lower in IL-27R deficient mice. Splenic germinal centre B cells were decreased in these mice. In addition, both auto-reactive IgM and IgG2a antibody levels were significantly decreased. These data suggest an impairment of B cell immunity in IL-27R deficient mice. However, CIA also depends on T cells. To determine whether the impairment of B cell immunity in IL-27R deficient mice was B cell intrinsic, we used the T cell independent TNP-Ficoll immunisation model. Wild type and IL-27R deficient mice were immunised with TNP-Ficoll i.p. and sacrificed seven days later. IL-27R deficient mice had fewer splenic IgM and IgG3 plasma cells and lower serum TNP specific IgM and IgG3 antibody levels.

Conclusions Here we show that IL-27R signalling on B cells is essential for proliferation and activation of B cells. Impaired B cell immunity in IL-27R deficient mice is caused by an intrinsic B cell defect, leading to impaired plasma cell formation and antibody production in both T cell independent as well as T cell dependent immune responses.

A5.22 **MEMBRANE-BOUND AND SOLUBLE BAFF EXPRESSION BY HUMAN RHEUMATOID FIBROBLAST-LIKE SYNOVIOCYTES IN RESPONSE TO TLR STIMULATION**

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Background and Objectives B cell activating factors of TNF family (BAFF) is associated with the survival and maturation of B cells. BAFF is widely expressed in the rheumatoid arthritis (RA) synovium which is characterised by the presence of synovial niches of autoreactive B cells and sustain in situ autoantibody production. Importantly, B cell niches remain functional in the RA-SCID model in the absence of recirculating cells, suggesting that autocrine mechanisms support ongoing B cell activation in the RA synovium. BAFF exerts its functional role both as a membrane bound protein and in soluble form. Here we investigated whether resident stromal cells in the RA synovium, synovial fibroblasts (RASF), are capable of producing either forms of BAFF and thus contribute to local B cell activation.

Methods mRNA BAFF in RASF stimulated with TLR2, TLR3 and TLR4 ligands was assessed by quantitative Taqman PCR RA dermal fibroblasts (RADF) and osteoarthritis SF (OASF) were used as controls. The cytoplasmic, membrane bound and/or soluble forms of BAFF were investigated by 1) Western blot using total and membrane-enriched protein extracts, 2) flow cytometry, 3) ELISA and 4) immunocytochemistry.

Results In vitro stimulation of TLR3, and to a significantly lesser extent TLR4, but not TLR2 on RASF led to strong induction of BAFF mRNA. In response to TLR3, soluble BAFF was time-dependently released in the supernatant of RASF (~600 pg/ml) and, to a lesser extent, OASF and RADF. RASF constitutively expressed both cytoplasmic and membrane bound BAFF as demonstrated by WB, FACS and immunocytochemistry which was upregulated upon TLR3 stimulation and was significantly increased as compared to RADF.

Conclusions Here we provide conclusive evidence that SF in the RA synovium are a pivotal source of the B cell survival factor BAFF at both mRNA and protein level. In addition to their significant constitutive expression, RASF can further up-regulate cytoplasmic, membrane-bound and soluble BAFF in response to TLR3 stimulation. Overall, our data strongly support a fundamental role for RASF in sustaining functional B cell activation and antibody production in the inflamed RA synovium.

A5.23 **MULTIPARAMETER PHOSPHO-FLOW ANALYSIS OF B CELLS FROM PATIENTS WITH RHEUMATOID ARTHRITIS**

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Background and Objectives Rheumatoid arthritis (RA) is a common, relapsing autoimmune disease, which affects approximately 1% of the population worldwide. While the specific molecular events that lead to initiation and onset of RA are not known, an uncontrolled activation of the immune system is considered to be a critical component of the disease. B lymphocytes undoubtedly play a critical role in disease aetiology. Antigen binding to B-cell receptor (BCR) triggers B cell activation, although the threshold of activation can be influenced by other receptors, such as TLR9. TLR9 has received substantial attention as a pathogenic co-stimulator of

autoreactive B cell responses. Our aim was to compare basal activity and induced phosphorylation of AKT, ERK, p38 MAPK and CREB after stimulation of B cells from RA patients and healthy individuals via BCR and/or TLR9.

Materials and Methods Blood samples were collected from healthy donors and RA patients having moderate (DAS28 3.2 < 5.1) and active (DAS28 > 5.1) disease. B cells were stimulated with anti-Ig (Fab')$_2$ and/or CpG ODN. Naive and memory B cells were identified by anti-CD20-A647 and anti-CD27-PE Phosphorylation level of AKT, ERK, p38 and CREB was detected before and after the stimuli by specific phospho-antibodies using multiparameter phospho-flow analysis. Results were evaluated by the FlowJo software.

Results The basal level of phosphorylation of signalling molecules was significantly higher in RA patients as compared to healthy donors. The induced level of phosphorylation was also higher in RA samples in most of cases, CpG stimulated memory B cells from patients with active disease have shown the highest values. In anti-Ig plus CpG ODN stimulated samples phosphorylation of all molecules was significantly higher in both naïve and memory RA B cells as compared to healthy controls. However, when compared to unstimulated cells, the increment of phosphorylation in the stimulated cells was the same or lower in RA samples.

Conclusions We have shown differences in the activation state of AKT, ERK, p38 and CREB in B cells from healthy individuals and RA patients. The higher basal phosphorylation level indicates the activated state of RA B cells. The lower capability of activation-induced phosphorylation may be a result of lower responsiveness of RA B cells. The analysis of phosphorylation signature in RA B-cells may provide new information to a better understanding of the disease.

A5.24 NEUTROPHIL GRANULOCYTES RESPOND TO SURFACE-BOUND IMMUNE COMPLEXES CONTAINING ANTI-TYPE II COLLAGEN ANTIBODIES FROM RA PATIENTS

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Background and Objectives We have earlier shown that surface-bound immune complexes containing anti-type II collagen antibodies (anti-CII IC) from rheumatoid arthritis (RA) patients and anti-CII IC stimulate monocyte proinflammatory cytokine production, associated with an acute onset RA phenotype. Anti-CII IC in joint cartilage are exposed to cells in the synovial fluid (SF). Neutrophil granulocytes are the major cell type in SF, where they co-localise with mononuclear cells (MNC). The objective was to investigate whether also granulocytes respond to anti-CII IC, and whether such a response was dependent on interaction with other cells in SF.

Materials and Methods An anti-CII RA serum together with human native collagen (CII) was used to create surface-bound anti-CII IC. Heparinised blood from 8 healthy donors was separated into neutrophil granulocytes (>95% purity) and MNC. For each donor, the granulocyte cell fractions as well as co-cultures (granulocytes + MNC) (0.5 × 10E6/ml of each cell fraction) was cultured on anti-CII IC as well as on negative control IC prepared with normal human serum on CII and in a positive control IC system with purified IgG coated onto plastic. After 18 hours, cells were harvested for the measurement of CD11b, CD66b, CD16 and CD32 on granulocytes by flow cytometry, and supernatant levels of TNF and IL-8 was measured by ELISA.

Results In granulocyte cultures both anti-CII IC and control IC induced significant up-regulation of CD11b and CD66b, and

significant down-regulation of CD16 and CD32. When the granulocytes were co-cultured with MNC, there was a significant increase in CD11b up-regulation and CD16 down-regulation than granulocytes, with no effect on CD32 and CD66b. In the co-culture system, the anti-CII IC-induced production of IL-8 was significantly increased, but no such difference was noted for TNF. Isolated granulocyte fractions produced very low levels of TNF and IL-8 after IC stimulation.

Conclusions Isolated granulocytes respond to RA anti-CII IC in a model system mimicking IC in RA cartilage. The granulocyte responses depend on interaction with MNC.

Our anti-CII dependent RA phenotype is a human counterpart to collagen antibody-induced arthritis. Strong granulocyte reactivity to anti-CII IC might therefore be related to the Ncf1 gene involved in NADPH activity important in collagen-induced arthritis models.

A5.25 PROTEOMIC ANALYSIS OF ANTI-CCP IMMUNOGLOBULINS FOR THE IDENTIFICATION OF RHEUMATOID ARTHRITIS PATIENTS THAT REQUIRE EARLY AGGRESSIVE TREATMENT REGIMENS

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Background and Objectives Antibodies against citrullinated peptides (ACPA) are a hallmark of rheumatoid arthritis (RA) patients and are associated with poor outcome. ACPA are usually detected using the anti-CCP test, identifying antibodies against a broad spectrum of citrullinated antigens. Most citrullinated antigens however appear to be bystander antigens that are not thought to be involved in the disease process. While controversy remains over the identity of the pathogenic antigen, it has been hypothesised that the fine-specificity of the ACPA response in the patient harbours prognostic properties. For this purpose sera will be studied from early RA patients using a novel technique that might reveal differences in that fine-specificity of the ACPA response.

Materials and Methods We recently showed that the antibody response to a particular antigen results in rearranged immunoglobulin segments that are shared between individuals exposed to that antigen. In patients with paraneoplastic neurological syndromes, such sequences were found after proteomics analysis of affinity enriched immunoglobulins. In the current study, ACPA positive RA sera were drawn from 58 patients participating in the tREACH-study. This is a study with a protocolised treatment regime for patients with early RA aimed at obtaining low-disease activity (DAS < 2.4). Poor outcome was defined as the need for treatment with anti-TNF to control disease activity. Sera were affinity enriched using CCP2 ELISA plates, and the specific IgG was analysed by LC-MS.

Results Up to 1 μg of CCP specific IgG was obtained from 180 μl of serum. The LC-MS data was analysed for correlations between ACPA derived peptides and the need for treatment with anti-TNF to control disease activity. However, no such correlations were found in excess of the false discovery rate in this dataset. In addition to ACPA, we also affinity purified rheumatoid factors from sera as a control. These preparations could be distinguished from the ACPA, suggesting our method performed appropriately.

Conclusions Our experiments could not show a significant difference between anti-CCP antibodies in early RA sera from patients with different disease outcome. While this could indicate that anti-CCP fine-specificities are not associated with disease outcome,