

## B cells and autoantibodies: diagnosis and pathophysiology

### Oral presentation

#### 001 TRANSMEMBRANE BAFF FROM RHEUMATOID SYNOVIOCYTES, COUPLED WITH IL-6, INDUCES THE EXPRESSION OF RECOMBINATION-ACTIVATING GENE (RAG) IN NORMAL B CELLS

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**Background:** Rheumatoid arthritis (RA) is the most frequent chronic inflammatory disorder leading to major changes in synovial tissue (ST) with fibroblast like synoviocyte (FLS) activation and hyperplasia, and major inflammatory infiltration. Lymphoid cells in the ST may be diffuse or arranged in germinal centres (GCs). The V(D)J gene combinations are revised in the germinal centres by the recombination-activating gene (RAG) 1 and RAG2 enzymes, provided their B cell expression is coordinate. The present work was designed to gain insight into the mechanisms regulating expression of RAGs in B-cells and their functionality in secondary Ig rearrangements in RA.

**Methods:** We elaborated a model of co-culture of normal peripheral B cells with RA-FLS, to induce RAGs expression. Using single cell PCR and immunofluorescence analysis we evaluated RAGs expression due to RA-FLS contact at the mRNA and protein level. Using an LM-PCR analysis to detect Jk and/or Jλ gene rearrangements, we explore the ability of RAGs to induce V(D)J rearrangements. We explore the role of IL-6 in this model using ELISA measurement and co-culture with IL-6 antibody and the role of BAFF using BAFF-deficient RA-FLS generated by short hairpin (sh) gene interference mechanism.

**Results:** We present mRNA and protein evidence that fibroblast-like synoviocytes (FLS) from rheumatoid arthritis (RA), but not from osteoarthritis (OA), induce the expression of RAG in normal B cells. This expression is turned off by engagement of the BCR. One feature in the RAG expression is the transmembrane form of BAFF on RA-FLS. Consistent with this, shRNA downregulates the synthesis of BAFF preventing thereby RA-FLS to trigger RAG, and this failure is not reverted by rBAFF. However, the fact that OA-FLS do not induce RAGs, despite their expression of BAFF, suggests that another required feature is furnished by FLS from RA, but not from those from OA. We suspect IL-6, on the basis that Ab blockade of its receptor prevents RA-FLS to induce RAG, and, conversely, that rIL-6 provides OA-FLS with the capacity to trigger the expression of RAG by normal B cells.

**Conclusion:** Our findings suggest that BAFF-mediated cell-cell contact, coupled with the production of IL-6 by RA-FLS, induces V(D)J rearrangements and could lead to auto-immunity in RA.

### Oral presentation

#### 002 RITUXIMAB PREFERENTIALLY DEPLETES ACTIVATED B-CELLS BOTH IN THE PERIPHERY AND BONE MARROW IN RHEUMATOID ARTHRITIS PATIENTS

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**Objective:** Bone marrow is an immunologically privileged site where pathogenic B cells may survive for prolonged periods of time and escape depleting therapies. We investigated the effect of Rituximab in both peripheral blood and bone marrow B-cell populations of RA patients.

**Methods:** Peripheral blood (PB) and bone marrow (BM) aspirates from RA patients were collected at baseline and 3 months after Rituximab treatment. Patients were treated with 1000 mg of Rituximab on days 1 and 15. Measurement of B and T-cell populations was assessed with flow cytometry after isolation of the peripheral blood and bone marrow mononuclear cell fraction. Lymphocyte subpopulations were defined as follows: naïve B-cells (CD19+CD27-); memory B-cells (CD19+CD27+); plasmacytoid cells (CD19+CD27+CD38+); activated B-cells (HLA-DR+ CD19+); CD3+, CD4+ T cells; activated T cells: CD3+DR+, CD3+CD25 or CD3+CD69+. Disease activity was assessed by the DAS28 index. Absolute number of cells could only be estimated for the peripheral blood but not for the bone marrow since the latter is diluted by the presence of peripheral blood.

**Results:** The percentage of B cells (CD19+) decreased both in the periphery (1.6% to 0.8%, n = 7) and at the bone marrow (10% to 4%, n = 7) although the latter did not reach statistical significance (p = 0.058 and p = 0.2 respectively). Of interest at both sites the percentage of activated B cells was markedly decreased. More specifically a comparable reduction of CD19+DR+ was observed both within the BM compartment (76% to 20%, n = 7, p = 0.02) and the PB (from 63% to 8%, n = 7, p = 0.03). There was a trend towards decrease in the percentage of naïve B cell population in both compartments while plasmacytoid cells remained stable throughout the course of follow-up. Of note, baseline absolute B cell number was low (36.6 ± 31 cells/μL) probably reflecting the effect of previous therapies. Rituximab modestly decreased absolute B cell number in peripheral blood (36.6 ± 31 cells/μL to 18.1 ± 17.8 cells/μL, p = 0.047, n = 7) with a concomitant increase in memory B cells (212 cells/μL to 1185 cells/μL, p = 0.068).

After therapy, CD3+ T cells increased in the BM compartment (57% to 77%, n = 7, p = 0.063) while they remained stable in the periphery. No significant effects were observed on the activation status of T-cells within the BM.

Disease activity reduced significantly at four and six months (p = 0.02 and p = 0.011 respectively) as well as rheumatoid factor levels (p < 0.001). No correlations were observed between reductions in B-cell numbers and disease activity or rheumatoid factor.

**Conclusion:** Rituximab depleted activated B cells both in the periphery and the BM compartment in RA patients, while no consistent effect was observed on T cell activation status.

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### Oral presentation

#### 003 HIGH LEVELS OF SERUM ANTI-PEPTIDYLARGININE DEIMINASE 4 ANTIBODIES ARE ASSOCIATED WITH DISEASE SEVERITY AND PREDICT RADIOGRAPHIC PROGRESSION DESPITE ANTI-TNF-α THERAPY IN ESTABLISHED RHEUMATOID ARTHRITIS PATIENTS

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**Background:** Antibodies targeting citrullinated proteins are specific for rheumatoid arthritis (RA). Antigen citrullination *in vivo* is catalyzed by the Peptidylarginine Deiminase (PAD) enzyme family, most likely the PAD isoforms 2 and/or 4. Critical enzymes are often targeted by disease-specific antibodies in complex immune-mediated diseases. We have previously demonstrated raised levels of serum anti-human recombinant PAD4 (anti-hPAD4) IgG in Caucasian RA patients compared to SLE patients and healthy controls. Here, we further assessed anti-hPAD4 IgG in 123 RA sera, and analyzed for associations between high anti-hPAD4 IgG levels and disease severity as well as radiographic progression.

**Methods:** A time-resolved fluorometric immunoassay based on hPAD4 was developed and used to analyze RA sera obtained at baseline and after one year. 83 patients had recent onset RA, with disease duration less than one year. For 40 patients with established RA (mean disease duration 11.5 years), anti-TNF- $\alpha$  therapy was initiated at baseline (Adalimumab; 13 patients, Infliximab; 15 patients or Etanercept; 12 patients). We analyzed for differences in laboratory, clinical and radiographic damage variables across baseline anti-hPAD4 quartiles in the recent onset RA cohort and between pooled quartiles in the established RA cohort.

**Results:** Anti-hPAD4 IgG levels were stable in both RA cohorts, except for a few patients undergoing seroconversion. Serum anti-hPAD4 IgG levels were higher in the established RA cohort than in the recent onset RA cohort. In the recent onset RA cohort, we found no differences in laboratory, clinical or radiographic damage variables across anti-hPAD4 quartiles. In the established RA cohort however, we found higher swollen and tender joint counts and elevated DAS28 in the high anti-hPAD4 pooled quartile compared to the low anti-hPAD4 pooled quartile ( $p=0.02$ ,  $p=0.05$  and  $p=0.05$ , respectively). Patients with high anti-hPAD4 levels seemed to accumulate radiographic erosions despite anti-TNF- $\alpha$  therapy, expressed by progression in the van der Heijde modified Sharp (vdHSharp) erosion score at 6 and 12 months compared to baseline. The patients were dichotomized into groups with and without progression in the vdHSharp erosion score at 6 and 12 months. We found higher percentages of patients with radiographic progression at 6 and 12 months in the high anti-hPAD4 group compared to the low anti-hPAD4 group (7% vs 50%,  $p=0.03$  and 13% vs 64%,  $p=0.02$ , respectively).

**Conclusion:** Anti-hPAD4 IgG can be detected in RA sera and the levels are stable, also for patients receiving anti-TNF- $\alpha$  therapy. For recent onset RA, the importance of serum anti-hPAD4 IgG is not clear. However, our data suggest that high anti-hPAD4 IgG levels are associated with RA severity and predict radiographic progression in patients with established disease receiving anti-TNF- $\alpha$  therapy.

## Oral presentation

### 004 THE RHEUMATOID ARTHRITIS-SPECIFIC AUTOANTIBODIES TO CITRULLINATED PROTEINS COMPLEXED WITH FIBRIN(OGEN) INDUCE MACROPHAGE SECRETION OF TNF- $\alpha$ THROUGH ENGAGEMENT OF FC $\gamma$ RIIA

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**Objective:** Macrophage-derived tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a dominant mediator of synovitis in rheumatoid arthritis (RA) but the factors involved in its production are not completely elucidated. Autoantibodies to citrullinated proteins (ACPA) are specific for RA and probably play an important role in its pathophysiology. They are produced in the rheumatoid synovial tissue where their main target corresponds to citrullinated fibrin. We undertook to assess whether and how immune complexes (IC) formed by the interaction of ACPA with citrullinated fibrin deposits contribute to TNF- $\alpha$  production by synovial macrophages.

**Methods:** We developed an *in vitro* human model in which macrophages, derived from monocytes of healthy blood donors, were stimulated with ACPA-containing IC generated by capturing ACPA from RA sera on immobilized citrullinated fibrinogen. Cellular activation was evaluated by TNF- $\alpha$  assay in culture supernatants. Selective blockades of IC interactions with the three classes of Fc $\gamma$  receptors (Fc $\gamma$ R) served to assess their respective contribution to macrophage activation. Finally, two previously described citrullinated fibrin-derived peptides bearing major ACPA

epitopes,  $\alpha$ 36-50cit<sub>38,42</sub> and  $\beta$ 60-74cit<sub>60,72,74</sub>, were tested for their capacity to inhibit formation of macrophage-activating ACPA-containing IC.

**Results:** Monocyte differentiation into macrophages was accompanied by a significant induction of CD206 and Fc $\gamma$ RIII expressions in a very high proportion of the cells, a significant reduction in the percentage of Fc $\gamma$ RI-positive cells and no change in the proportion of Fc $\gamma$ RII-positive cells. ACPA-containing IC induced a dose-dependent TNF- $\alpha$  secretion by macrophages from 14 out of 20 healthy donors. Macrophage response was systematically higher than that of the paired monocyte precursors. TNF- $\alpha$  secretion was not reduced by blockade of Fc $\gamma$ RI or Fc $\gamma$ RIII but strongly repressed if interaction of IC with Fc $\gamma$ RII was prevented. The two citrullinated peptides significantly inhibited ACPA reactivity to citrullinated fibrinogen and, used together, almost completely abolished formation of macrophage-activating IC and hence TNF- $\alpha$  secretion.

**Conclusion:** The totally human *in vitro* model we developed to study the interaction between macrophages and ACPA-containing IC has allowed demonstration of its inflammatory potential via engagement of Fc $\gamma$ RIIa at the surface of macrophages, strongly supporting the pathophysiological involvement of ACPA. Further use of this model should permit molecular dissection of the interaction and of its functional consequences as well as to explore how they could be modulated. The capacity of both the  $\alpha$ 36-50cit<sub>38,42</sub> and  $\beta$ 60-74cit<sub>60,72,74</sub> peptides to substantially inhibit the reactivity to C-FBG of a highly polyclonal mixture of ACPA confirms that both represent major epitopes recognized by ACPA on this antigen and represent highly interesting potential tools for future immunotherapeutic strategies.

## Oral presentation

### 005 PROTECTIVE CROSSREACTIVITY OF RHEUMATOID FACTORS WITH B CELL STIMULATORY ssDNA STIMULATES TLR9 AND MAY BE INVOLVED IN ARTHRITIS INDUCTION

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**Objective:** Mice unable to express DNase II, a DNA-degrading enzyme residing in phagocytic cells, develop a disease that closely resembles human rheumatoid arthritis (RA). Many serological parameters, including production of rheumatoid factors (RF) and antibodies to citrullinated peptides, are also consistent with human RA. The status of RA patients with respect to DNase I and II gene expression is presently unknown, as well as the contribution of Rheumatoid factors (RF) to arthritis, and the Toll-like 9 receptor with its role in promoting RF production and inducing the disease in humans.

**Results:** Gene expression of DNase I and II was analyzed using DNA microarrays with representational difference analysis. Synovial tissue expression of DNase I was very low in RA patients and control synovial tissue; DNase II was expressed but was similar or only slightly enhanced by a factor of 1.6 in 47% of the RA patients studied. Affinity purified RF specifically inhibited ssDNA degradation and bound to K type B cell stimulatory oligonucleotides that stimulate IL-6 production, but not to D type oligos which stimulate IFN $\alpha$  production by natural killer cells. Moreover, RF is able to protect non-methylated cytosine guanine dinucleotide-containing oligonucleotides (a typical component of bacterial DNA, or mitochondrial DNA) from degradation which is known to activate macrophages and TNF-dependent joint inflammation. Using hypomethylated mitochondrial DNA, 8oxodG containing oligonucleotides we could efficiently stimulate RA B cells for RF production, in contrast to using PHA. Interestingly, RF, but not anti-citrulline antibodies were induced by non-methylated oligonucleotides in PBMCs or B cells from RA patients.

**Conclusion:** The status of RA patients with respect to DNase I and II gene expression is presently unknown, but we show here for the first time that there is no significant change in DNase I or II expression in RA patients compared to controls. Co-stimulation of the B cell receptor and TLR9 appears to represent an effective mechanism to induce RF-IgM in RA patients and RF cross reacts and protects K type B cell stimulatory oligonucleotides. These new data indicate that ssDNA might be protected by RF to further stimulate Toll 9 and induce arthritis.

## 006 TRANSGLUTAMINATED AND CITRULLINATED PROTEINS ARE TARGETED BY RHEUMATOID ARTHRITIS PATIENT SERA

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**Purpose:** Protein modification can generate neopeptides from self proteins, causing autoaggressive immune attack. We found that transglutaminase 2 (tTG2) is highly expressed in synovial tissue of rheumatoid arthritis (RA) patients. We have now investigated this topic in more detail.

**Methods:** Using the technology of the RZPD providing protein filters (27000 Proteinfilter) the autoantigen profile of citrullinated and transglutaminated proteins could be obtained. Moreover citrullinated proteins from serum exosomes have been identified by 2D and mass spectroscopy. We used an IgG IgA-anti-tissue transglutaminase antibody (anti-tTg) ELISA to assess the prevalence of anti-tTG, in synovial fluid from 68 patients with RA. Articular chondrocyte cultures obtained from adult human donors were used for gliadin peptide stimulation studies. For immunohistochemical analyses, 1–3-µm paraffin sections of synovial tissue obtained from patients with RA, patients with osteoarthritis (OA), and normal were used.

**Results:** Out of 27000 citrullinated or transglutaminated human proteins we found 101 citrullinated and 64 transglutaminated proteins reactive with RA patients sera. Interestingly the same 22 proteins are only reactive when transglutaminated or citrullinated. Previously identified citrullinated autoantigens (vimentin, fibrinogen) were found as well as proteins which are involved in TGF β binding and regulation. Looking at the anti-tTG2 response in synovial fluid from 68 RA patients we found that 38% had anti-tTg IgG/IgA, 15% had anti-IgA tTg but only 5% of the osteoarthritis group had anti-IgA tTg. Gene expression of tTGs and PADs was analyzed using DNA microarrays with representational difference analysis. Synovial tissue expression of tTG 2 was enhanced by a factor of 1.6 in 57% of the RA patients studied. Using a monoclonal anti-tTG 2 we found tTG2 significantly overexpressed in RA synovial tissue compared to normal or OA tissue. Certain transglutaminated modified gliadin peptides were able to induce IL-6 and IL-8 in primary chondrocytes. This induction of IL-6 and IL-8 was TLR receptor independent. Moreover, the autoimmune autoantigen profile was compared to expression profiles of these autoantigens in synovial membrane from RA patients and controls, showing that modification and not differential mRNA expression may be a central mechanism for development of autoreactivity.

**Conclusion:** In this high throughput approach we identified new citrullinated and transglutaminated antigens targeted by RA patients sera. Two of the proteins have also been identified in serum exosomes from RA patients and are involved in TGF β binding and regulation. Transglutamination might therefore provide a means of provoking an autoaggressive immune response as a result of food intake or infection. This would be an alternative to immune activation by self-mimicking microbial antigens, for which definitive proof remains elusive in human disease.

## 007 THE AUTOANTIBODY RESPONSE IN VERY EARLY RHEUMATOID ARTHRITIS IS DIRECTED AGAINST ENZYMES OF THE GLYCOLYTIC PATHWAY AND MOLECULAR CHAPERONINS: ROLE OF CITRULLINATION

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The aim of our study was to identify new proteins and their post-translational modifications (PTMs) targeted by the very early rheumatoid arthritis (RA) autoimmune response. Sera obtained from 110 early untreated RA patients and 20 healthy volunteers were analyzed by Western blot using HL-60 cell extract, separated on 1- and 2-dimensional gel electrophoresis (1-, 2-DE). The immunoreactive proteins were identified by MALDI-TOF mass spectrometric analysis and the presence of potential sites of citrullination in each of these proteins was evaluated. The 110 1-DE patterns isolated 10 recurrent immunoreactive bands of 33, 39, 43, 46, 51, 54, 58, 62, 67 and 70 kDa which were further characterized by 2-DE and proteomic analysis. Six proteins corresponded to already-described RA autoantigens: hnRNP A2/B1, aldolase, alpha-enolase, calreticulin, HSP60 and BiP. Phosphoglycerate kinase1 (PGK1), stress-induced phosphoprotein1 and the far upstream element-binding proteins (FUSE-BP) 1 and 2 were identified as new autoantigens. PTMs were observed for most of these proteins, notably aldolase, alpha-enolase, PGK1, calreticulin, HSP60 and the FUSE-BPs possessed potentially deiminated peptides. Anti-CCP antibodies were significantly associated with the reactivity of RA sera against p39, p46, p58 and p62. A reactivity against 3 peptides corresponding to alpha-enolase, HSP60 and FUSE-BP 1 was significantly associated with the presence of autoantibodies to p46, p62 and p67. The glycolytic enzyme family and molecular chaperonins such as FUSE-BP 1 appear to play an important role in the very early RA autoimmune response. Their citrullination, which notably occurs in vivo in haematopoietic cells, might be involved in their antigenicity and thus in the development of the autoimmune response directed against citrullinated peptides which characterizes RA.

## 008 THE TYPE I IFN SIGNATURE DETERMINES THE SUSTAINED ANTI-CITRULLINATED PROTEIN ANTIBODIES LEVELS DURING TNFα BLOCKADE IN RHEUMATOID ARTHRITIS

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**Objective:** Increasing evidence points towards a crucial role of type I interferons (IFN) in humoral autoimmunity in diseases such as systemic lupus erythematosus and Sjögren syndrome. A type I IFN signature was also recently described in rheumatoid arthritis (RA) (Van der Pouw-Kraan, ARD, 2007) but the relationship with RA-specific autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) is unknown. As these antibodies are differentially regulated during TNF blockade in vivo (De Rycke, ARD, 2005) and as TNFα neutralization sustains IFNα production in vitro, we investigated the role of type I IFN in the regulation of autoantibody titres both at baseline and during TNF blockade.

**Patients and methods:** Serum was collected from 21 RA patients before and after 24 weeks of infliximab treatment. The type I IFN signature was determined by peripheral blood gene expression analysis at baseline. ACPA IgG and IgM, RF IgM, anti-dsDNA IgM and anti-nucleosome IgM were measured by ELISA.

**Results:** Nine patients had a type I IFNlow signature and 12 patients had a type I IFNhigh signature. At baseline, RF IgM, ACPA



IgM, and ACPA IgG levels were not associated with IFN type I signature in RA. RF IgM was significantly down modulated by TNF blockade (33.3 U/ml versus 20.7 U/ml,  $p = 0.0016$ ) irrespectively of the baseline type I IFN signature and the changes in disease activity score (DAS). In contrast, ACPA IgG levels were exclusively down modulated in the type I IFNlow group (611 U/ml versus 344 U/ml,  $p = 0.023$ ) but remained stable in the type I IFNhigh group (272 U/ml versus 245 U/ml). This was not related to the isotype of the autoantibodies as ACPA IgM were similarly decreased in the type I IFNlow group (7.4 relative units/ml versus 5.11 relative units/ml,  $p = 0.008$ ) but not in the type I IFNhigh group (7.5 relative units/ml versus 9.9 relative units/ml). As for RF, the changes in ACPA IgM and IgG were not related to response to therapy as reflected by the changes in DAS. Finally, this modulation is specific for pre-existing ACPA as the de novo induction of anti-dsDNA and anti-nucleosome IgM by infliximab was similar in type I IFNlow and type I IFNhigh RA patients.

**Conclusions:** The baseline type I IFN signature determines the persistence of ACPA levels during TNF $\alpha$  blockade, pointing to a specific regulation of ACPA in comparison with other autoantibodies in RA.

TC and CW contributed equally to this study

#### 009 ABERRANT RAG EXPRESSION IN SYSTEMIC LUPUS ERYTHEMATOSUS B-CELLS IS DUE TO OVEREXPRESSION OF IL-6

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**Background and aims:** Different checkpoints regulate expansion of autoreactive cells during B cell development. Though defective regulation of V(D)J recombination's promotes the production of autoantibodies in systemic lupus erythematosus (SLE), the central tolerance checkpoint in the bone marrow appears to be effective in most human patients with SLE. A second peripheral checkpoint could thus be defective, as suggested in mice where defect in secondary Ig gene rearrangements in mature peripheral B-cells may be instrumental in the development of the disease. The aim of the present study was to evaluate the ability of the B cell receptors (BCR) of peripheral B lymphocytes to turn off the recombination-activating gene (RAG) 1 and RAG2 in this disease.

**Patients and methods:** In 20 SLE patients and 17 healthy controls, B cells were purified, RAG1 and RAG2 mRNA expression analysed by RT-PCR before and after in vitro stimulation of the BCRs with anti-IgM antibodies, and cellular RAG-2 protein localization determined by confocal microscopy. Expression of CDK2 and p27Kip1, negative and positive regulators of RAG2 respectively, were also evaluated by flow cytometry and western blotting. Levels of IL-6 in the culture supernatants were measured by an enzyme-linked immunosorbent assay, and its activity evaluated using a blocking anti-IL-6 receptor antibody.

**Results:** Patients with SLE showed a high frequency of RAG-positive peripheral B lymphocytes compared with healthy controls. Furthermore, BCR engagement induced a shift in the frequency of kappa- and lambda-positive cells, associated with a persistence of RAG mRNA expression and the maintenance of the RAG2 protein within the nucleus. While CDK2 expression was found to be normal, that of p27Kip1 was up regulated, and further enhanced following BCR engagement. Moreover, only B cells from SLE patients were spontaneously able to produce IL-6, the level of which was further increased after BCR stimulation. Interestingly, elevated levels of p27Kip1, and subsequent nuclear retention of RAG2, depended on the secretion of IL-6 by B cells. Indeed, blockade of IL-6 activity induced a down-regulation of p27Kip1 expression, and allowed the translocation of RAG2 from the nucleus to the cytoplasm.

**Conclusion:** Our findings highlight a novel function for IL-6. Therefore, due to their inability to terminate RAG expression following BCR engagement, in association with constitutive IL-6 signalling, the SLE B cells would become prone to Ig gene recombination and, in turn, to autoantibody production.

#### 010 IMPAIRED DNA METHYLATION IN SYSTEMIC LUPUS ERYTHEMATOSUS (LED) B-CELLS IS RESTORED USING ANTI-IL-6 RECEPTOR (R) ANTIBODY (AB)

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**Purpose:** Epigenetic regulation is a reversible mechanism by which genes can be silenced. Several studies have highlighted the importance of DNA methylation in lupus, and led to the observation that DNA methyltransferase (DNMT) regulation is impaired in lymphocytes. Thus, based on the fact that IL-6 controls DNMT activity, we reasoned that IL-6 overexpression in B-cells may contribute to epigenetic aberrancies in SLE.

**Methods:** Peripheral blood B-cells were purified from 10 SLE patients and 15 healthy controls. The levels of mRNA for DNMT1 and IL-6 were assessed by quantitative RT-PCR. Bisulfite DNA sequencing and methylation-sensitive endonuclease (Hpa II) followed by PCR enabled us to evaluate the methylation status of CD5-E1B promoter used as control previously shown to be under DNMT regulation. B-cells were stimulated in vitro with anti-IgM Ab, in the presence or not of IL-6 and blocking anti-IL-6 R Ab.

**Results:** Activation of the B-cell receptor with anti-IgM Ab induces an opposite effect in patients and controls. In the patients, no variation could be detected in DNMT1 mRNA level, while that of IL-6 was strikingly upregulated ( $\times 710.226.7$ ). Analysis of the CpG motifs in CD5-E1B promoter revealed that upon stimulation, new CpG motifs are demethylated. In the controls, DNMT1 was upregulated ( $\times 19.12.5$ ), associated with a slight increase of IL-6 ( $\times 15.23.4$ ) and CpG motifs are methylated. To evaluate the influence of an autocrine effect of IL-6 on DNMT1 expression, SLE B-cells were stimulated in presence of anti-IL-6-R Ab. In this condition, DNMT1 was induced ( $\times 58,913.5$ ) and CpG demethylation impaired.

**Conclusions:** Excess of IL-6 in activated SLE B-cells represses DNA methylation. IL-6 signalling blockade can restore DNMT1 activity, calling for the clinical relevance of the anti-IL-6-R Ab in the treatment in SLE.

#### 011 INTERLEUKIN 21 (IL-21) IS INCREASED IN PATIENTS WITH ACTIVE SYSTEMIC LUPUS ERYTHEMATOSUS AND MAY ACT SYNERGISTICALLY WITH TLR-9 TO PROMOTE B-CELL ACTIVATION AND AUTOANTIBODY PRODUCTION

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**Aim:** Excessive production of interleukin 21 (IL-21) results in lupus-like disease in mice. We have reported an increased percentage of TLR-9 expressing memory and plasma cells correlating with the presence of anti-dsDNA antibodies in patients with active SLE and an increased synergistic effect of TLR-9 and IL-21 towards B-cell differentiation into memory B-cells and plasma cells in healthy individuals. To explore the relative contribution of these two pathways in the differentiation of B cells in SLE we measured IL-21 and IL-21 receptor (IL-21R) in SLE patients and healthy controls and compared the synergistic effect of the two pathways on the differentiation of B cells in the two populations.

**Materials and methods:** The expression of IL-21 and IL21 receptor (IL-21R) was measured using real time PCR in peripheral blood

mononuclear cells (PBMCs) of lupus patients and healthy controls. Isolated B-cells from healthy donors or lupus patients were stimulated using combinations of IL-21 and TLR-9 or TLR-3 ligands (ODN 2006, poly I:C) in the presence or absence of hydroxychloroquine and the differentiation of B-cells into memory cells (CD19+CD27+) or plasma cells (IgD-CD38high) was evaluated using flow cytometry.

**Results:** Interleukin 21 (IL-21) was increased in active SLE patients compared with inactive patients or healthy controls (4-fold increased relative expression in active SLE ( $n = 12$ ) vs inactive SLE ( $n = 11$ ,  $p = 0.002$ ) or healthy controls ( $n = 10$ ,  $p = 0.003$ )). The expression of IL-21R did not differ among groups under study. Interestingly, IL-21 with either TLR-9 or TLR-3 ligands exhibited an enhanced ability of promoting B-cell differentiation to plasma cells in active compared to inactive lupus patients (17% increase in plasma cells with ODN+IL-21 in active SLE patients ( $n = 7$ ) vs 4.5% increase in inactive patients ( $n = 6$ ,  $p = 0.05$ ) and 17.5% increase in plasma cells with poly I:C+IL-21 in active SLE patients ( $n = 7$ ) vs 4.16% in inactive patients ( $n = 6$ ,  $p = 0.01$ )). Experiments addressing the synergistic effects of IL-21 and TLR ligands on B-cell activation and autoantibody production in SLE are in progress.

**Conclusions:** Toll-like receptors recognizing self derived nucleic acids may act synergistically with IL-21, a cytokine that is overexpressed in active SLE, to promote B cell differentiation and autoantibody production in SLE.

#### 012 ANTI-dsDNA AUTOANTIBODIES BUILD IMMUNE-COMPLEXES WITH NUCLEAR FRAGMENTS IN WHOLE BLOOD AND MEDIATE INFLAMMATION IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

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**Objective:** Deficiencies in the recognition and phagocytosis of dead and dying cells have been shown to be one of the main alterations in patients with systemic lupus erythematosus (SLE). Cellular as well as humoral elements play an important role in the clearance of apoptotic and necrotic cells. Autoantibodies against nuclear antigens of the IgG isotype may influence the clearance of dead and dying cells in patients with SLE by binding its cognate antigen in circulation.

**Methods:** We established a whole blood ex vivo phagocytosis assay comprising cellular and humoral components. This enabled the concomitant monitoring of the uptake by granulocytes and monocytes of various particulate targets.

**Results:** We found an impaired uptake by granulocytes and monocytes from patients with SLE of both, IgG-opsonised and albumin-coated polystyrene beads. However, the uptake of yeast particles and bacteria was not significantly different from that of normal healthy donors (NHD). Surprisingly, the uptake of nuclear fragments was increased in most patients with SLE and correlated with antibodies against double stranded DNA. Nuclear fragments were not phagocytosed in whole blood of NHD. The transfer of IgG fractions from SLE to NHD whole blood induced increased phagocytosis of nuclear fragments. Inflammatory cytokines were produced after the uptake of nuclear fragments by non-professional phagocytes.

**Conclusion:** The whole blood phagocytosis assay represents a "close to the in vivo picture" of the patients' actual clearance status in blood. Some patients with SLE show selective defects in the phagocytosis of certain targets. Opsonisation by the patients' antibodies recognizing double stranded DNA of nuclear fragments fosters its uptake by monocytes and granulocytes. We describe here

how the antibody-mediated clearance drives an amplification loop of the chronic inflammatory response that may contribute to the maintenance of autoimmunity. Interventions in this inflammatory vicious cycle could represent a new effective approach for the treatment of patients with SLE.

#### 013 HIGH MOBILITY GROUP BOX PROTEIN 1 (HMGB1)-NUCLEOSOME COMPLEXES FROM APOPTOTIC CELLS INDUCE ANTI-dsDNA ANTIBODIES IN NON-AUTOIMMUNE MICE: IMPLICATIONS FOR ETIOPATHOGENESIS OF SYSTEMIC LUPUS ERYTHEMATOSUS

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Pathogenic autoantibodies against dsDNA and nucleosomes (NCs) represent a hallmark of systemic lupus erythematosus (SLE). However, the factors leading to the autoimmune response against these poorly immunogenic nuclear components are not fully identified. We suggest that high mobility group box protein 1 (HMGB1), a nuclear DNA-binding protein as well as a secreted pro-inflammatory mediator, may represent an endogenous adjuvant leading to the breakdown of immunological tolerance. During primary necrosis, HMGB1 is passively released and induces an inflammatory response. During apoptosis, however, HMGB1 gets tightly attached to hypoacetylated chromatin and is not released, since apoptotic cells are immediately engulfed by phagocytes. We investigated if HMGB1-nucleosome complexes which may be released from secondary necrotic cells in conditions with impaired clearance of apoptotic cells, as it has been shown in a subgroup of SLE patients, can exert an inflammatory response and induce autoantibodies to dsDNA. We found that HMGB1 remains bound to NCs released from apoptotic cells in vitro. Also in sera and plasma of some patients with SLE, but not in controls, complexes of HMGB1 and NCs were detected. Importantly, HMGB1 containing NCs from apoptotic cells induced secretion of cytokines including IL-1 $\beta$ , IL-6, IL-10, and TNF $\alpha$  as well as expression of costimulatory molecules on human macrophages and dendritic cells (DC), respectively. Neither HMGB1-free NCs from living cells nor from apoptotic HMGB1-deficient cells induced marked cytokine production or DC activation. Specific inhibition of HMGB1 activity by the antagonistic A box domain significantly reduced capacity of "apoptotic" NCs to induce TNF $\alpha$  and IL-10 release by macrophages. Importantly, immunization with NCs from apoptotic cells induced significant anti-dsDNA and anti-histone IgG responses in non-autoimmune BALB/c and C57BL/6 mice, whereas NCs from living cells did not.

We conclude that HMGB1-NCs complexes released from apoptotic cells activate antigen presenting cells, thereby contributing to autoimmunity against nucleosomes/dsDNA and the immunopathogenesis of SLE.

#### 014 ALTERATION OF B CELL PHENOTYPE FOLLOWING DEPLETION WITH RITUXIMAB IN RHEUMATOID ARTHRITIS

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**Background:** B cell depletion using Rituximab (RTX) is now established for the treatment of rheumatoid arthritis (RA). We previously explored the extent of B cell depletion in blood and synovial tissue. Blood depletion is profound (from two weeks post-treatment) and lasts until approximately 26 weeks post-treatment. Synovial B cell depletion is correlated with synovial T cell depletion and reduction in disease activity. Another study has shown that synovial B cell depletion precedes changes in other cell types.<sup>1</sup>

Studies in SLE have suggested that RTX therapy modifies B cell activation and antigen presenting cell function. This study, therefore, examined expression of markers of B cell activation, cross-talk, antigen presentation and survival in RA, to determine whether these B cell functions may be modified post-RTX therapy.

**Methods:** 12 patients were treated with 2 infusions of 1g RTX. Blood samples were obtained pre-infusion and 6, 14 and 26 weeks post-infusion. Expression of cell surface markers was analysed using flow cytometry. B cells were identified by CD19 expression, and their expression of CD32, CD40, CD80, CD86, HLA DR and BAFF receptor (BAFF R) was measured.

**Results:** At baseline, B cells formed a relatively homogenous population, expressing low levels of CD80 and CD86, high levels of CD40 and HLA DR, with two subpopulations of CD32 (positive and negative) and two BAFF R populations (high and low). Expression of CD40 was reduced at 6 weeks compared to pre-treatment, increased between 6 and 26 weeks, but remained lower than at baseline. HLA DR expression followed the same pattern, with reduction at 6 weeks, maintained at 26 weeks. Expression of CD86 increased between 14 and 26 weeks; however, this was associated with the emergence of a separate population of cells from those which initially remained post-treatment. CD32 expression decreased between baseline and 6 weeks post-treatment, but had increased to pre-treatment levels by 14 weeks. BAFF R expression followed a similar pattern; however, at 26 weeks it was significantly higher than at baseline.

**Conclusions:** These data suggest that B cells which repopulate the circulation following RTX therapy are different in phenotype from B cells pre-treatment and could, therefore, be involved in breaking the cycle of co-stimulation and cross-talk with T cells. This is in agreement with data indicating the co-depletion of B and T cells in the synovium. BAFF R expression appears to be important in the survival and maturation of these cells. Further work is required, to determine whether these are newly developed naïve B cells or surviving B cells which have been immuno-modulated, and whether differential B cell subset repopulation could provide an insight into the clinical response to RTX treatment.

1. Vos, et al. *Arthritis Rheum* 2007;**56**:772–8.

# 015 MARKED DIFFERENCES IN FINE-SPECIFICITY AND ISOTYPE USAGE OF THE ANTI-CITRULLINE RESPONSE IN HEALTH AND DISEASE

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**Background:** Anti-citrullinated protein antibodies (ACPA) display a high association with rheumatoid arthritis (RA) and have been implicated in its pathogenesis. We have characterized this antibody response, for the first time, in a unique population of RA patients and their unaffected relatives, and identified the features that could confer its pathogenicity.

**Methods:** IgA, IgM and IgG ACPA, as well as IgM and IgA rheumatoid factor (RF), were measured in serum from 76 RA patients and 208 unaffected relatives.

The isotype distribution, fine-specificity of the ACPA response, and its association with RF were compared in health and disease.

**Results:** Around 1/5 of the healthy relatives were positive for ACPA. The frequency of all ACPA isotypes was strikingly lower in unaffected relatives than in RA patients: most patients were positive for all 6 ACPA isotypes, while relatives were positive for 1 isotype, maximum 2. Fine-specificity studies showed that reactivity to citrullinated fibrinogen and vimentin was present in the RA sera, while it was virtually absent in unaffected relatives. Finally, the

ACPA and RF responses associated in the RA patients, while being discordant in healthy relatives. Extended analyses revealed that ACPA was associated with RA irrespective of RF status, while the association of RF with disease relied on its interaction with ACPA.

**Interpretation:** Fine-specificity and isotype usage of the ACPA response are qualitatively different in health and disease. Epitope spreading and expansion of the isotype repertoire might be necessary for development of RA and this could be facilitated by the presence of RF antibodies.

# 016 CLINICAL FEATURES OF 11 PATIENTS AFFECTED BY SYSTEMIC SCLEROSIS WITH ANTI-RNA POLYMERASE III ANTIBODIES

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**Aim:** To define clinical features of patients affected by systemic sclerosis (SSc) with antibodies to RNA polymerase III.

**Methods:** Anti-nuclear autoantibody profile in 427 patients affected by SSc, followed by our Unit, was studied. Every serum was analysed by IIF and counterimmunoelectrophoresis. Anti-topoisomerase-I antibodies were found in 23.2%, anti-centromere in 47.5%. Fifty-two available sera, out of 117 sera anti-topoisomerase-I and anti-centromere negative, were tested for anti-RNA polymerase III (RNA-pol), using commercial ELISA (MBL).

**Results:** Eleven anti-RNA-pol positive patients (mean age at onset: 50 y, SD: 19 y) were affected by diffuse cutaneous SSc in 9 cases (81.8%), with a rapid disease onset: they show a median interval of 2 months between the onset of Raynaud's phenomenon and the first symptom of SSc. Interstitial lung disease and pulmonary arterial hypertension were detected in 72.7% and 27%, respectively. A severe skin involvement with joint contractures was reported in 8 cases (73%), while digital ulcers were found in 6 patients. Cutaneous and gastro-intestinal teleangiectasias were detected in about one third of cases. The rapid onset of the disease or the development of lung involvement had required the use of immunosuppressive treatment in six patients (54.5%). Comparing 11 anti-RNA pol positive patients with 94 anti-topoisomerase positive patients, we did not find any significant difference in age at onset, sex distribution and rate of diffuse SSc type. The same rate of lung involvement was recorded between the two groups (73% vs 82%), with a comparable mean value of FVC and DLCO at onset. Otherwise, we observed a severe worsening of DLCO during follow up in anti-topoisomerase positive group ( $p = 0.0008$ ), while it seemed stable in anti-RNA pol subjects. SSc renal crisis rarely occurred in both groups (9% in anti-RNA pol positive patients and 6.3% in anti-topoisomerase-I positive cases). Surprisingly, a water-melon stomach and enteric angiectasias were found only in patients with anti-RNA pol antibodies (27.7% of cases). Anti-RNA polymerase-III antibodies showed an antinuclear speckled pattern in 8 cases, nucleolar pattern in 2 sera and speckled with nucleolar staining in one case. No other circulating autoantibody was found in association with anti-RNA polymerase-III.

**Conclusions:** Anti-polymerase III positive patients showed a severe SSc, characterised by pulmonary and diffused skin involvement, with a rapid onset of the disease. We did not find a higher rate of scleroderma renal crisis, comparing these patients with anti-topoisomerase I SSc. According to other authors, anti-RNA polymerase III did not show a strict nucleolar staining on HEp-2 cells.

# 017 B-CELL PHENOTYPICAL AND TRANSCRIPTIONAL ANALYSIS IN SALIVARY GLANDS OF PATIENTS WITH SJÖGREN'S SYNDROME

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**Background:** Based on their predominance within lacrimal and salivary glands (SG) of Sjögren's syndrome (SS), T cells have long



been at the forefront of the pathophysiology. That B lymphocytes also are activated has been established, but their role restricted to the production of antibodies. The major aim of this study was to delineate B cell-subpopulations participating in the lymphocyte infiltrate of SG from patients with SS. A special emphasis was placed on those B cells included in the ectopic germinal centre (GC).

**Methods:** Their phenotype was thoroughly analyzed using a number of double-colour combinations. A transcriptional analysis has also been performed using real-time RT-PCR after laser microdissection of the B cell infiltrates and compared with normal tonsils.

**Results:** Using immunofluorescence, we show that a very limited number of B cell infiltrates fulfil the criteria for ectopic GC, i.e. the GC phenotype tonsil (CD20<sup>+</sup>, IgD<sup>+</sup>, CD38<sup>-</sup>, CD21<sup>-</sup> and CD24<sup>+</sup> in the mantle zone, and CD20<sup>+</sup>, IgD<sup>-</sup>, CD38<sup>+</sup>, CD21<sup>+</sup> and CD24<sup>-</sup> in the GC). Indeed, most of the pseudo-GC lacked characteristics of centroblasts and centrocytes. Instead, they are reminiscent of transitional type 2 B cells, and alike marginal-zone (MZ) B cells (CD20<sup>+</sup>, IgD<sup>+</sup>, CD38<sup>-</sup>, CD21<sup>+</sup> and CD24<sup>+</sup>). Like tonsil GC, phenotypically-defined GC express *Pax-5*, *Bcl-6* and *AICDA*, while other infiltrated-B lymphocytes, like MZ B lymphocytes, express *Blimp-1* and *Notch-2*.

**Conclusion:** Ectopic GC do exist in a minority of SG, but the majority consist of transitional type 2 and MZ-like B cells. These should be instrumental in the local production of autoantibodies and the ensuing destruction of epithelial cells in SS.

#### 018 FINE SPECIFICITY OF THE ANTI-CITRULLINATED PROTEIN ANTIBODY RESPONSE AT DIFFERENT STAGES OF THE DISEASE COURSE OF RHEUMATOID ARTHRITIS

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**Objective:** Anti-citrullinated protein antibodies (ACPA) are the most predictive factor for the development and progression of rheumatoid arthritis (RA). In this study, we investigated if the antigen recognition pattern of the ACPA response continues to evolve during disease progression.

**Methods:** Antibodies against the five best-described citrullinated antigens (derived from vimentin, fibrinogen and alpha-enolase) were determined by enzyme-linked immunosorbent assay in sera of anti-cyclic citrullinated peptide (CCP) positive patients. Epitope recognition patterns at baseline of patients with undifferentiated arthritis (UA) who later developed RA (UA-RA) were compared to those of patients who did not progress to RA (UA-UA). ACPA reactivities of UA-RA patients after development of RA were compared to baseline values. To investigate the development of ACPA specificities during a longer period of follow-up, baseline serum samples from RA patients were compared to sera from the same patients obtained 7 years later.

**Results:** At baseline, UA patients who later developed RA recognized significantly more peptides than UA-UA patients. At later stages of the disease course, there was no expansion of epitope recognition, but instead a decrease in the levels of epitope-specific antibodies. The number of peptides recognized at follow-up was comparable to the number recognized at baseline.

**Conclusion:** These data indicate that anti-CCP positive UA patients who will develop RA already differ from those who will not with regards to their ACPA reactivities at the time of disease onset, and furthermore that broadening of the recognition pattern of citrullinated antigens takes place during an early stage of the disease.

#### 019 C-REACTIVE PROTEIN (CRP), SERUM AMYLOID P COMPONENT (SAP) BIND TO hnRNPs AND OTHER RNA OR DNA BINDING AUTOANTIGENS: IDENTIFICATION OF A SHORT POLYPEPTIDE BINDING REGION

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C-reactive protein binds autoantigens and itself is a target in SLE patient sera; it is of great interest which proteins are bound by CRP and what proteins when targeted can induce loss of the protective IL-10 production, rendering it to an immune complex mediated interferon alpha response. Elevated IFN- $\alpha$  and overexpression of interferon-induced genes have been observed in SLE patient blood and correlate with disease severity. CRP binds to chromatin, histones, and small nuclear ribonucleoproteins (snRNPs). To study the interaction of CRP to other potential autoantigens we used a protein filter binding assay to study calcium dependent and independent binding.

**Methods:** Using the technology of the RZPD providing protein filters (27000 human Proteinfiler) far western blot analysis with CRP was performed. Recombinant hnRNPs and peptides (Dot Blot) of hnRNPs were used to study binding to CRP and SAP. To further define the binding specificity the proteins were separated by 15% SDS-PAGE and transferred on nitrocellulose and probed with monoclonal mouse anti-CRP, SAP.

**Results:** In total 233 clones, mostly calcium dependent CRP binding proteins, were identified. 56 represent identified full length clones: chromatin associated proteins, histone associated proteins, splicing factors (SmD), SR proteins, enzymes, surface proteins, autoantigens, and a protein in a genetic susceptibility locus for rheumatoid arthritis. CRP binds to hnRNP A2/B1, an A1 previously shown to be targeted by RA and SLE patient sera. In order to identify the target region for binding we used peptides from the hnRNP A/B proteins. The CRP binding region could not be identified but a high homologous RGG rich region was identified as the binding region of SAP to hnRNP A1/A2/B1/A3 which turned out to be calcium independent. A comparison of the protein sequences from the identified CRP binding proteins show homologies to previously identified arginine glycine rich binding motif. The binding region from CRP to hnRNPs and other identified CRP binding proteins need to be identified. These reactions were inhibited by EDTA, implicating the calcium-dependent binding of CRP to hnRNP A/B proteins.

**Conclusion:** In this study we have demonstrated the capacity of CRP to interact with more than 50 different proteins, mainly DNA and ssRNA, dsRNA binding proteins in a solid protein filter format. Moreover hnRNPs autoantigens in RA and SLE have been found to interact with CRP and a small peptide was identified to bind SAP. This region contains several RGG motifs but is not bound by CRP. Autoantibody reactivity to bound autoantigen-CRP complex in vivo might render its activity from protection to autoimmunity. Moreover it might explain why hnRNPs and other autoantigens are targeted in SLE and RA patients independent from the amount of CRP in the sera of the patients. DNA-containing ICs inducing IFN- $\alpha$  might explain lower CRP concentrations in SLE and patients with viral infections.

#### 020 AUTOANTIBODIES IN RHEUMATOID ARTHRITIS: ANTI-CITRULLINATED PROTEIN ANTIBODIES (ACPA) AND TYPE II COLLAGEN SPECIFICITIES

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Several autoantibodies have been reported to be associated with rheumatoid arthritis, RA e.g. type II collagen (CII) antibodies have long been implicated early in disease, and by using three triple helical peptides, CII specificity can be revisited. Additionally,

alpha-enolase has recently been reported as a candidate antigen in RA. We were interested in measuring reactivity against these proteins. For this purpose, we have used the EIRA cohort. EIRA is a unique material comprised of a large number of RA patients and population based controls that are well characterized with regard to genetic, environmental and clinical factors. In the present study, subjects were divided into aCCP+ patients (n=483), aCCP- patients (n=228) and healthy controls (n=150). This material belongs to the same cohort that has been used for the genome wide study which was recently published in *N Engl J Med*.<sup>1</sup>

Our study demonstrates that more patients are reactive to the different peptides among aCCP+ patients, than among aCCP-. Still, the aCCP- group displayed higher frequencies than the healthy controls. In the aCCP+ group both cit- $\alpha$ -enolase and cit-C1-CII antibodies were common with 65% and 49% frequency, respectively. Also, the aCCP+ cohort showed reactivity towards non-citrullinated CII peptides (C1 and U1), but these were lower than towards citrullinated CII C1. Double reactivity against the two cit-peptides C1 and  $\alpha$ -enolase were associated with higher aCCP levels among aCCP+ patients. Moreover, there was correlation between reactivities against citrullinated peptides and the presence of shared epitope, which was not seen for the non-citrullinated peptides.

Determination of antibody reactivity is presently ongoing for citrullinated fibrinogen and vimentin, in parallel with relating all the serological results to the genetic parameters recently defined in these cohorts.<sup>1</sup>

1. Klareskog, Gregersen, et al. TRAF1-C5 as a risk locus for rheumatoid arthritis--a genomewide study. *N Engl J Med* 2007;**357**:1199–209.

## 021 DIAGNOSTIC AND PROGNOSTIC VALUE OF IGG AND IGA-ANTIBODIES AGAINST CYCLIC CITRULLINATED PEPTIDES (ANTI-CCP) AMONG EARLY RHEUMATOID ARTHRITIS PATIENTS (RA)

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**Objective:** Antibodies against citrullinated peptides (anti-CCP) are routinely analyzed only concerning IgG. Earlier studies have shown that IgA RF both can prognosticate severe disease and predict future development of RA. We therefore wanted to investigate IgA anti-CCP in RA patients.

**Methods:** We measured IgG and IgA anti-CCP in a cohort of 270 early RA patients and in 100 healthy control sera. The results were correlated with clinical data obtained at baseline and during five years follow-up.

**Results:** At inclusion, 166/270 (61.5%) of patients and 0/100 (0%) of healthy control were positive for IgG anti-CCP while 173/270 (64%) of patients were IgA anti-CCP positive (defined as 95th percentile of controls). Of the 104 IgG anti-CCP negative patients, 29 were IgA anti-CCP positive, resulting in a total sensitivity of 72% (195/270) for IgG and/or IgA anti-CCP.

The differences between IgG anti-CCP positive and negative patients became more obvious with time especially physicians assessment of disease activity, swollen joints, tender joint counts and DAS28. The corresponding differences were less prominent for IgA anti-CCP. In all these cases anti-CCP positive patients had worst prognosis. Among IgG anti-CCP-negative patients IgA anti-CCP was instead significantly associated with better prognosis for every variable except ESR and CRP. IgA anti-CCP was instead associated with higher ESR.

**Conclusion:** The additional diagnostic contribution of IgA anti-CCP might be clinically important. Prognostically IgA anti-CCP has a dichotomous role, alone associated with low disease activity but

together with IgG anti-CCP associated with further high disease activity.

## 022 LEVELS OF SERUM AMYLOID A AND ITS AUTOANTIBODIES IN AUTOIMMUNE DISEASES WITH AND/OR WITHOUT THROMBOSIS

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**Background:** Serum amyloid A (SAA) is an acute phase protein, markedly increased during acute and chronic inflammation in animals and humans, and its levels are elevated in autoimmune diseases. SAA has been indicated to represent a prediction marker for sub-acute stent thrombosis and a prognostic marker for increased mortality in acute myocardial infarction patients. There is a lack of information concerning the level of antibodies against SAA (anti-SAA) and their association with SAA protein in human sera.

**Aims of study:** Our goal was to investigate anti-SAA levels in sera of autoimmune patients with and without venous and/or arterial thrombosis, and to compare the levels of anti-SAA antibodies with the levels of SAA, CRP and IL-6.

**Materials and methods:** Sera from healthy blood donors (n = 29) and patients groups (n = 84) with set diagnoses were compared in their levels of SAA, CRP and IL-6 by ELISA. In order to determine the levels of anti-SAA autoantibodies, an in house ELISA was adapted from a previously developed anti-SAA ELISA method. Our small, pilot, cross-sectional study involved an optimization of ELISA methodology for the detection of anti-SAA in autoimmune patients, while comparing their levels to venous and arterial thrombotic patients.

**Results:** Two different human recombinant apo-SAA antigens gave relevant differences in our ELISA results. The saturation thresholds of the two antigens were different, as were the sera responses. The measurements above 99% percentile from the blood donor group (n = 29) were considered as positive. The highest number of anti-SAA positives was found in the systemic lupus erythematosus with antiphospholipid antibodies group (40%) of patients and in systemic lupus erythematosus with antiphospholipid syndrome (17%), whereas the venous and arterial thrombotic groups of patients exhibited absorbances lower than those of blood donors.

**Conclusions:** There was no correlation found between anti-SAA and SAA levels (n = 97), nor with CRP, and only slight correlation between anti-SAA levels and IL-6 levels in the tested sera. It would be interesting to search for correlations of anti-SAA with cardiovascular involvement, since the highest number of positives come from either SLE in the presence of antiphospholipid antibodies or SLE in combination with APS. In future, the numbers of patients within the groups would need to be further expanded, which could lead to the elucidation of potential biological implications of anti-SAA.

## 023 AN ANALYSIS OF THE B CELL RESPONSE

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There is a growing body of evidence suggesting a role for B lymphocytes as key players in the inflammatory process of rheumatoid arthritis. To analyse the immune response of synovial B cells we are using Laser Capture Microdissection (Arcturus). B cell infiltrates and plasma cells were microdissected directly from frozen tissue sections, RNA was extracted and the H- and L-chain genes of the immunoglobulins amplified. A sequence analysis of V-region genes showed that both naïve and memory B cells immigrate into the inflamed synovial tissue of patients with rheumatoid arthritis. However, in T-B cell infiltrates only memory B cells are activated. They differentiate into plasma cells which are found associated



with infiltrates, but also lining the vasculature. The analysis of V-gene sequences indicated that there is class switch, but no evidence of hypermutation. Somatic diversification of V-region genes seems to take place only in germinal centres. We are now determining the specificity of synovial naïve, memory and plasma cells. It will show whether the activation of B cells in synovial infiltrates and their differentiation into plasma cells is antigen dependent.

#### 024 ANTI-CITRULLINATED PROTEIN ANTIBODIES (ACPA) AND ANTIBODIES TO EXOGENOUS ANTIGENS IN FOLLOW-UP STUDY OF RHEUMATOID ARTHRITIS PATIENTS

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Antibodies to citrullinated proteins (ACPA) are a marker of rheumatoid arthritis (RA). Several data suggest a peculiar regulation of their production: ACPA of the IgM isotype are in fact detectable in RA patients several years after the disease onset. The predictive value of these antibodies in terms of disease activity is still a matter of debate.

The aim of our work is to analyze the modification in titer of ACPA and antibodies to exogenous antigens, correlating such modifications with the activity of the disease in a follow-up study of RA patients.

We measured the levels of anti-CCP, anti-VCP, anti-deiminated fibrinogen, anti-EBNA I and anti-tetanus toxoid antibodies in blood samples collected from 30 RA patients in two visits at least 4 months apart. A full clinical and serological evaluation was concomitantly obtained for each patient. The modification of antibody levels was expressed as a ratio between the values obtained at the end and at the beginning of follow-up.

The levels of different ACPA were highly correlated ( $p < 0.0001$ ), as well as the magnitude of their variation ( $p < 0.01$ ). ACPA did not correlate with antibodies to exogenous antigens, either in levels or in variation. In our study ACPA or antibodies to exogenous antigens did not show any correlation with disease activity (expressed as CRP and ESR levels).

Our data confirm that antibodies to different citrullinated proteins belong to a single antibody family, characterized by similar frequency, levels and variations in time. As previously reported, ACPA are not a reliable marker for disease activity in RA patients. The lack of correlation between the different antibody families and disease activity markers suggests that antibody production is not affected by the severity of inflammation. Moreover, our work indicates that ACPA production is regulated differently from the production of antibodies to chronic-infection or recall antigens.

#### 025 THE INCREASE OF TYPE I IFN SERUM ACTIVITY BY TREATMENT WITH ETANERCEPT, BUT NOT INFlixIMAB, DOES NOT INFLUENCE THE INDUCTION OF ANTI-NUCLEAR ANTIBODIES IN SPONDYLOARTHRITIS

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**Objective:** We reported previously that TNF blockade in spondyloarthritis (SpA), an immune-mediated disease without known autoantibodies, leads to the induction of non-pathogenic, IgM anti-nuclear antibodies (De Rycke, A&R, 2003). Surprisingly, this induction was much more pronounced with infliximab than with etanercept, indicating different mechanisms of action of both TNF blockers (De Rycke, A&R, 2003). As increasing evidence points towards a crucial role of type I interferons (IFN) in humoral autoimmunity in systemic lupus erythematosus and as TNF $\alpha$  neutralization sustains IFN $\alpha$  production in vitro, we investigated here if type I IFN activity is modulated by anti-TNF $\alpha$  treatment and thereby influences the induction of anti-nuclear antibodies.

**Patients and methods:** We collected serum of SpA patients during treatment with either infliximab ( $n = 20$ ) or etanercept ( $n = 20$ ). Type I IFN serum activity was determined by IFN-inducible gene expression after incubation of the WISH cell line with patient serum. Anti-dsDNA IgM, anti-nucleosome IgM, BAFF, and APRIL serum levels were measured by ELISA.

**Results:** Baseline type I IFN serum activity in SpA was in the range of healthy controls. During infliximab treatment, type I IFN serum activity was transiently downmodulated at week 2 ( $p = 0.005$ ) but returned to baseline levels at later time points (week 6 and 12). In contrast, etanercept treatment induced a persistent upregulation of type I IFN serum activity ( $p < 0.03$  from week 4 to week 12). Accordingly, there was a significant difference in changes of type I IFN serum activity between the infliximab and etanercept treatment over a 12 weeks period ( $p = 0.036$ ). In contrast to type I IFN, factors involved in B lymphocyte maturation and survival such as BAFF and APRIL were unaffected by TNF blockade. As reported previously, there was a significant induction of anti-dsDNA IgM ( $p = 0.03$ ) and anti-nucleosome IgM ( $p = 0.02$ ) in the infliximab cohort but not the etanercept cohort. However, the induction of these antibodies was neither correlated with the baseline type I IFN serum activity nor associated with the changes of type I IFN during treatment.

**Conclusion:** In SpA, treatment with etanercept, but not infliximab, increases type I IFN serum activity, pointing towards different mechanisms of actions of both drugs. However, the modulation of type I IFN serum activity can not explain the preferential induction of anti-nuclear antibodies by infliximab rather than etanercept.

#### 026 NEW AUTOANTIGENS IN RHEUMATOID ARTHRITIS: SCREENING PROTEIN ARRAYS WITH SERA FROM PATIENTS WITH DIFFERENT HLA-DR GENOTYPES

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**Objective:** To determine whether specific HLA-DR genotypes are associated with specific autoantibody production patterns in rheumatoid arthritis.

**Methods:** We tested serum samples from 19 rheumatoid arthritis (RA) patients with given HLA-DR genotypes, be they very high risk (DRB1\*0401/0404), very low risk (DR7/DR7), or very unexpected (like the high risk DR7/DR9), from 7 spondyloarthritis patients, from 2 lupus patients, from 4 systemic sclerosis patients, and from 10 healthy individuals on protein arrays containing more than 8000 human proteins.

**Results:** Four antigens were identified that exhibited enhanced reactivity from sera in RA patients relative to control sera. The differential reactivity of four antigens will be confirmed using ELISA assays. Our most striking result is that 47% of RA patients are positive for human PAD4 (peptidyl arginine deiminase 4) versus 0% of spondyloarthritis patients, 0% of lupus patients, 0% of systemic sclerosis patients and 0% of healthy individuals.

**Conclusion:** Screening protein arrays containing more than 8000 human proteins with sera from patients demonstrated that PAD4 is a specific autoantigen in rheumatoid arthritis.

## T cells in autoimmunity

### Oral presentation

#### 027 IL-23 IS CRITICAL IN THE DEVELOPMENT OF A NON-AUTOIMMUNE MODEL OF ARTHRITIS AND IS ESSENTIAL FOR IL-17 PRODUCTION BY TCR $\gamma\delta$ T CELLS IN THE SPLEEN AS WELL AS IN THE SYNOVIUM

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It has been shown that IL-23 plays a critical role in the development of autoimmune collagen-induced arthritis. IL-23p19 deficient mice

(IL-23KO) were fully protected and did not develop CIA at all. This was accompanied with collagen-specific CD4<sup>+</sup> IFN $\gamma$ <sup>+</sup> cells but no CD4<sup>+</sup> IL-17<sup>+</sup> cells. Furthermore, a recent study showed high IL-17 production in CIA mice by TCR $\gamma\delta$  T cells.

However, it is unknown whether IL-23 is involved in the development of joint inflammation and tissue destruction in a non-autoimmune model of arthritis and whether IL-23 regulates IL-17 production by TCR $\gamma\delta$  T cells in AIA.

**Objective:** To examine the role of IL-23 in the development of chronic joint inflammation and bone erosion in a non-autoimmune, antigen-induced arthritis (AIA) model. Also, the role of IL-23 in IL-17 producing CD4<sup>+</sup> and TCR $\gamma\delta$  T cells was assessed.

**Results:** Macroscopic analysis revealed mono-arthritis in wildtype mice (wt) with a maximal joint inflammation at day 7, which stayed high till day 10. However, IL-23KO developed significantly milder arthritis with a maximum at day 2 which declined to almost normal at day 10. Histological analysis revealed mild joint inflammation in IL-23KO similar to wt at day 1. In contrast, at day 7 severe joint inflammation and bone erosion was observed in wt, which was significantly suppressed in IL-23KO. Production of TNF, IFN $\gamma$  and MCP-1 was significantly reduced in the synovium of IL-23KO compared to wt mice. No significant suppression of synovial IL-6 levels was noted in the absence of IL-23. Of note, synovial IL-17 protein expression was reduced in IL-23KO at days 1, 2, 7 and 10, indicating lower activation of IL-17 producing T cells. To address this further, splenic CD4<sup>+</sup> and TCR $\gamma\delta$  T cells were isolated from AIA mice and analyzed for intracellular cytokine expression. CD4<sup>+</sup> IL-17<sup>+</sup>, but not IFN $\gamma$ <sup>+</sup> T cells were markedly decreased in IL-23KO compared to wt at days 1 and 7. Interestingly, IL-17<sup>+</sup> and IFN $\gamma$ <sup>+</sup> TCR $\gamma\delta$  T cells were detected in wt at day 1 and were increased at day 7. In contrast, IL-17<sup>+</sup> TCR $\gamma\delta$  T cells but not IFN $\gamma$ <sup>+</sup> TCR $\gamma\delta$  T cells were markedly decreased in IL-23KO compared to wt at days 1 and 7. In addition, IL-17 production by synovial TCR $\gamma\delta$  T cells was decreased at day 7 compared to day 1 in wt and was hampered by the lack of IL-23.

**Discussion:** These data show a critical role of IL-23 in the progression of chronic destructive arthritis in a non-autoimmune model of arthritis. Lack of IL-23 resulted in reduced synovial expression of inflammatory cytokines including IL-17. Furthermore, IL-17 production by TCR $\gamma\delta$  T cells is IL-23 dependent as observed in the spleen and in the synovium of AIA mice. These data suggest an important role for the IL-23/IL-17 immune pathway in the progression of experimental arthritis and indicates a critical role of IL-23 in IL-17 production not only in CD4<sup>+</sup> T cells but also in TCR $\gamma\delta$  T cells.

## Oral presentation

### 028 CD25+IL-7R- DEFINED FOXP3+ T HELPER CELLS ARE POTENT SUPPRESSORS OF IL-7R+ TH CELLS AND ARE INCREASED IN THE JOINTS OF RHEUMATOID ARTHRITIS PATIENTS

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**Purpose:** IL-7 is a potent immunostimulatory cytokine. Increased IL-7 levels are found in several inflammatory diseases including rheumatoid arthritis (RA). IL-7 effects are essentially mediated through the high affinity IL-7Receptor-alpha chain (IL-7R) in conjunction with the common-gamma chain. IL-7 stimulates proliferation, survival and differentiation of T cells and induces T cell dependent monocyte, B cell and osteoclast formation. Recently, it has been suggested that the lack of IL-7R expression on CD25+ T cells could help to identify "true" regulatory T cells (Tregs), expressing high FoxP3 levels. Our purpose was to investigate the association of joint inflammation in RA patients and numbers of these better defined Tregs and study the capacity of these cells to suppress function of arthritogenic T cells.

**Methods:** Numbers of CD4+ T cells expressing either CD25 or the IL-7R (CD127), co expressing or lacking both CD25 and the CD127, were assessed in paired samples of synovial fluid and blood from RA patients (n = 10). From the CD25CD127-defined T cell subsets FoxP3 expression was assessed as a marker of suppressive function. In addition, from these defined subsets the anergic state as well as the capacity to suppress CD25-CD127+ aggressive T cells were determined.

**Results:** CD25+ Tregs were significantly (at least p<0.05) increased in synovial fluid (SF) compared to peripheral blood (PB) (18 vs 10%). Of CD25+ cells 36% was FoxP3+, significantly higher compared to CD25- cells (4% FoxP3+), but not significantly different from SF CD25+ T cells (37%). Comparable to CD25+ cells, CD127- T cells consisted of a high percentage of FoxP3+ cells (30%), which were significantly increased in SF compared to PB (40 vs 15%). Interestingly, CD127+ T cells hardly expressed FoxP3 (4 vs 5% in PB vs SF, resp). Most important, we observed that PB CD25+ T cells lacking CD127 identified a T cell subset that consisted of 70% FoxP3+ cells. These cells were increased in SF compared to PB (p<0.001) with on average comparable expression FoxP3 levels. By contrast, CD25-CD127+ T cells, either from PB or SF, hardly expressed FoxP3. In line with the FoxP3 expression CD25+CD127- T cells were anergic and significantly more potent than either CD25+ or CD127- T cells in suppressing CD25-CD127+ proliferating T cells. Suppressive capacity of CD25+CD127- T cells from RA patients was not impaired compared to healthy controls.

**Conclusions:** Our data demonstrate improved identification of suppressive FoxP3+ Th cells by the presence of CD25 and the absence of CD127. These cells have similar FoxP3 levels in PB compared to SF and numbers of these cells are increased in SF. This suggests that joint inflammation in RA is not due to decreased numbers or decreased intrinsic suppressive function of CD25+CD127-Tregs. Furthermore, it is indicated that selective targeting of CD127+ T cells, which are largely FoxP3- and immunostimulatory, is a therapeutic option.

## Oral presentation

### 029 1,25-DIHYDROXYVITAMIN D3 MODULATES DEXAMETHASONE EFFECTS BY CHANGING TH1/TH17 AND TH2 CYTOKINE PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS IN EARLY RHEUMATOID ARTHRITIS PATIENTS

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**Background:** Corticosteroids are commonly used in the treatment of rheumatoid arthritis (RA), but also induce systemic bone loss. 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) stimulates bone formation and gives a shift from a pro-inflammatory into an anti-inflammatory immune response. It is unknown whether 1,25-(OH)2D3 may reduce corticosteroid induced bone loss by modulation of the cytokine balance in RA patients.

**Objective:** To study the effects of 1,25-(OH)2D3 and dexamethasone on the production of pro-inflammatory/pro-destructive Th1/Th17 cytokines IFN $\gamma$ , TNF $\alpha$ , IL-17A and the anti-inflammatory/anti-destructive Th2 cytokine IL-4.

**Methods:** Peripheral blood mononuclear cells (PBMCs) from 18 healthy controls and 18 untreated early RA patients were stimulated for 72 hours with antiCD3/antiCD28 in the absence and presence of various concentrations of 1,25-(OH)2D3, dexamethasone and 1,25-(OH)2D3/dexamethasone combined. IL-17A, TNF $\alpha$ , IFN $\gamma$  and IL-4 were measured in the supernatants by a specific ELISA.

**Results:** TNF $\alpha$  levels in supernatants of unstimulated PBMCs and IL-17A levels in supernatants of stimulated PBMCs were higher in early RA patients versus healthy controls. In both groups

1,25-(OH)<sub>2</sub>D<sub>3</sub> and dexamethasone significantly inhibited IL-17A and IFN $\gamma$  production. Dexamethasone also significantly inhibited the levels of TNF $\alpha$ . Interestingly, the combination of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and dexamethasone almost completely inhibited IL-17A and IFN $\gamma$  production. Furthermore, 1,25-(OH)<sub>2</sub>D<sub>3</sub> induced a three-fold increase in IL-4 and completely restored the IL-4 production that was inhibited by dexamethasone to levels as in supernatants of antiCD3/antiCD28 stimulated PBMC. 1,25-(OH)<sub>2</sub>D<sub>3</sub> decreased TNF $\alpha$ /IL-4, IL-17A/IL-4 and IFN $\gamma$ /IL-4 ratios, while dexamethasone increased TNF $\alpha$ /IL-4 and IFN $\gamma$ /IL-4 ratios. The unfavourable effect of dexamethasone on TNF $\alpha$ /IL-4 and IFN $\gamma$ /IL-4 ratios could be overcome by 1,25-(OH)<sub>2</sub>D<sub>3</sub>.

**Conclusion:** These data show a beneficial effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on the Th2 cytokine IL-4 and inhibition of the Th17 cytokine IL-17A. In addition, 1,25-(OH)<sub>2</sub>D<sub>3</sub> has an additional value on the inhibition of IL-17A by dexamethasone and restored the IL-4 production and the negative effect of dexamethasone on TNF $\alpha$ /IL-4 and IFN $\gamma$ /IL-4 ratios. These data suggest that 1,25-(OH)<sub>2</sub>D<sub>3</sub> may contribute to a bone sparing effect in RA patients using corticosteroids by modulation of the cytokine balance.

## Oral presentation

### 030 EARLY ACTIVATION OF NKT CELLS IN A RHEUMATOID ARTHRITIS MODEL AND ITS APPLICATION TO DISEASE TREATMENT

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**Background:** Invariant (i) NKT cells are a unique lymphocyte subtype, CD1d restricted, implicated in the regulation of autoimmunity, and a good source of various Th1, Th2 or Th17 cytokines. Activation of iNKT cells with their exogenous ligand  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) may exert a therapeutic effect in diseases such as rheumatoid arthritis (RA). However, their physiological role in RA, without any exogenous stimulation, is unclear.

**Objective:** To elucidate the physiological role of iNKT cells in collagen-induced arthritis (CIA), a model of RA in mice.

**Methods:** CIA was induced in DBA/1 mice by immunization with type II collagen in complete Freund adjuvant. On days 6, 22, 42 and 64, cytokines secretion was measured by multiplex assays, and expression of TCR V $\alpha$ 14-J $\alpha$ 18 mRNA was evaluated by real-time QRT-PCR in spleen, lymph nodes, blood and liver. iNKT cells were stained with an APC-conjugated  $\alpha$ -GalCer-loaded CD1d tetramer and analyzed by flow cytometry. FACS profile of the activation marker CD69 was also assessed on iNKT cells. For treatment of CIA, mice were treated intraperitoneally with 200  $\mu$ g of either blocking anti-CD1d mAb, or control rat IgG, on days 0, 3 and 7 post induction of CIA.

**Results:** We demonstrated that activation of iNKT mainly occurred in the early phases of the disease (6 days post induction) and was characterized, in the liver, by secretion of IL-4, IL-17, IFN $\gamma$ , TGF- $\beta$ , overexpression of invariant TCR V $\alpha$ 14-J $\alpha$ 18 mRNA, and CD69 increased expression. However, no difference in the expression of TCR and cytokines secretion was observed in others organs at any time. Importantly, anti-CD1d mAb early treatment induced a significant diminution of clinical scores of arthritis.

**Conclusion:** These findings suggest that, in the beginning of the disease, iNKT cells are activated and may contribute to the pathogenesis of arthritis and can therefore be considered as a therapeutic target. Studies of interactions between iNKT and others cells of the immune system such as dendritic cells (DC) are in progress to further characterize iNKT early activation in CIA.

### 031 DELINEATION OF THE EXPRESSION, FUNCTION, AND THE ROLE OF PROGRAMMED DEATH-1 (PD-1) IN SYSTEMIC LUPUS ERYTHEMATOSUS: A GENETIC, IMMUNOHISTOCHEMICAL, AND IMMUNOLOGICAL STUDY

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**Aim:** PD-1, a negative regulator of T-cell function, has an important role in maintenance of peripheral tolerance. A putative regulatory intronic polymorphism in the human PD-1 gene (PD1.3) is associated with increased risk for systemic lupus erythematosus (SLE). We determined the expression and the function of PD-1 in patients with SLE.

**Materials-methods:** We genotyped 242 patients and 256 age- and sex-matched controls for the PD1.3 single nucleotide polymorphism (SNP) by PCR-RFLP and tested its effect on gene transcription using transient transfection assays. Expression of PD-1 and its ligand PD-L1 were determined by flow cytometry in peripheral blood mononuclear cells and in an ex vivo model of autoreactivity against self-antigens (autologous mixed lymphocyte reaction, AMLR), and by immunohistochemistry in lupus nephritis renal biopsies. A cross-linker of PD-1 (PD-L1.Fc) was used to assess its effects on T cell proliferation and cytokine production.

**Results:** SLE patients had increased frequency of the PD1.3 SNP (frequency of risk allele A: 28.5% in SLE vs. 18.4% in healthy donors; odds ratio = 1.55, 95% confidence interval 1.06–2.28). PD1.3 was found to confer decreased transcriptional activity in transfection assays, while a patient homozygous for PD1.3 displayed reduced expression on T cells at baseline and upon stimulation. PD-1 was detected within the glomeruli and renal tubules of patients with lupus nephritis, while its ligand PD-L1 was expressed by the renal tubules of both patients and controls. In AMLR experiments, PD-1 induction on CD4+CD25+ T cells was defective in SLE patients compared to healthy donors. Activation of PD-1 suppressed the anti-CD3/anti-CD28-induced proliferation and cytokine production both in normal and lupus T cells; addition of serum from active SLE patients significantly reduced this effect.

**Conclusions:** PD1.3 is a regulatory polymorphism associated with decreased transcription. Importantly, aberrant expression and function of PD-1 may occur in human SLE. The expression of PD-1/PD-L1 in renal biopsies and during AMLR suggests a role of the pathway in maintaining peripheral T cell tolerance. Modulation of PD-1/PD-L1 represents an additional therapeutic target in SLE.

### 032 INTERLEUKIN 17 PROMOTES SURVIVAL OF RHEUMATOID ARTHRITIS FIBROBLAST-LIKE SYNOVIOCYTES VIA REGULATION OF SYNOVIOLIN EXPRESSION

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**Objective:** IL-17 has an important role in chronic synovial inflammation; however, less is known for synovial hyperplasia. Synoviolin is a novel E3 ubiquitin ligase implicated in synovial hyperplasia. The capacity of IL-17 to regulate synoviolin expression, apoptosis and proliferation in rheumatoid arthritis synoviocytes is not known.

**Methods:** Synoviolin expression was analysed by real-time RT-PCR and Western Blot. p65 NFkB or AP-1 activation was measured by Trans AM Transcription Factor Assay Kits. Apoptosis was detected by annexin V/propidium iodide staining and SS DNA apoptosis ELISA kit. IL-17 receptor A (IL-17RA), IL-17 receptor C (IL-17RC) or synoviolin inhibition was achieved by small interfering RNA (siRNA) or neutralizing antibodies. Arthritis scores, synoviolin expression and Tunnel staining were detected in IL-17R wildtype



and deficient mice during chronic streptococcal cell wall induced arthritis.

**Results:** IL-17A, IL-1 or LPS were more potent inducers of synoviolin compared to TNF and IL-17F. IL-17 induced sustained synoviolin expression over 24 h. SNP treatment induced RA FLS apoptosis associated with reduced synoviolin expression. IL-17 treatment of RA FLS significantly abrogated sodium nitroprusside (SNP)-induced apoptosis, and decreased IL-17-induced synoviolin expression, NF $\kappa$ B or AP-1 activation. Synoviolin RNA interference enhanced SNP-induced apoptosis, and decreased IL-17-induced synoviolin expression. Synovial hyperplasia was markedly reduced associated with decreased synoviolin expression and increased apoptosis in IL-17R deficient mice during chronic reactivated streptococcal cell wall induced arthritis.

**Conclusion:** IL-17 induction of synoviolin may contribute at least in part to RA FLS dysregulated apoptosis and cell growth. These results extend the role of IL-17 to synovial hyperplasia.

### 033 CD4<sup>+</sup>CD25<sup>+</sup> REGULATORY T CELLS DIRECTLY INHIBIT INFLAMMATION BY SHEDDING OF sTNFRII

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**Background:** CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg) are potent suppressors of effector T cell function. We have previously shown that Treg cells adoptively transferred into mice with collagen-induced arthritis, a primarily B-cell mediated autoimmune disease model, markedly slow disease progression by decreasing systemic mediators of inflammation (Serum Amyloid P and IL-6). Interestingly, effector T cell function and collagen type II-specific autoantibody responses remained unchanged. This anti-inflammatory effect was also observed in mice injected with complete Freund's adjuvant only. These findings suggest that Treg cells can directly inhibit inflammation independently of arthritis induction, possibly by secreting soluble anti-inflammatory mediators.

**Objectives:** TNF- $\alpha$  is a key mediator of inflammation and has been shown to downregulate Treg cell function. We hypothesized that Treg cells might secrete soluble TNF Receptors (sTNFR) to control inflammation and protect themselves from TNF- $\alpha$  induced loss of regulatory function.

**Methods:** Murine and human Treg (CD4<sup>+</sup>CD25<sup>+</sup> in mice and CD4<sup>+</sup>CD25<sup>high</sup> in human) and CD4<sup>+</sup>CD25<sup>-</sup> effector T cell populations were isolated by FACS-sorting and subjected to TCR/CD28-stimulation. sTNFR were measured in culture supernatants by ELISA. Supernatants from these experiments were used in functional assays using a TNF- $\alpha$  sensitive reporter cell-line (WEHI 164 clone 13). Treg cells obtained from TNFRII-knockout animals served as control in the murine system. In addition, Treg cells from wild-type and TNFRII knock-out animals were adoptively transferred in mice subjected to LPS-stimulation, and serum IL-6 levels were detected as measures of systemic inflammation.

**Results:** Expression and shedding of TNFRII was found to be a prominent feature of both murine and human Treg cells. Highly purified Treg cells shed TNFRII abundantly upon activation in vitro, whereas CD4<sup>+</sup>CD25<sup>-</sup> effector T cells did so much less. Treg-derived sTNFRII was biologically active, as it could prevent TNF- $\alpha$  induced death of WEHI-cells. In addition, Treg cells from wild-type mice could efficiently inhibit LPS-induced IL-6 production in vivo, whereas Treg cells derived from TNFRII knock-out animals could not. The capacity of Treg cells from knock-out animals to suppress effector T cell function in a conventional suppression assay, however, was unaltered.

**Conclusion:** Our results reveal a novel functional feature of murine and human Treg cells. We show that Treg cells shed sTNFRII in biologically relevant amounts, and that Treg cell derived sTNFRII is able to control inflammation in a murine adoptive transfer system. These data could be relevant for Treg cell function in RA-patients, as the production of physiologic, Treg cell derived TNF- $\alpha$

antagonists could protect Treg from TNF- $\alpha$  induced functional impairment and regulate inflammation locally in the joint.

### 034 INCREASED IL-7 EXPRESSION IN SALIVARY GLANDS OF pSS PATIENTS CORRELATES WITH IMMUNOPATHOLOGY AND DRIVES TH1-ASSOCIATED IMMUNE RESPONSES

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**Purpose:** Evidence is accumulating that IL-7 contributes to inflammation in several chronic inflammatory diseases. Increased levels are found in diseases such as RA, juvenile idiopathic arthritis (JIA), psoriasis and psoriatic arthritis. IL-7 stimulates proliferation, survival and differentiation of T cells. In addition, IL-7 induces both T cell-dependent and T cell-independent cytokine secretion by monocytes. These prominent immunoregulatory effects indicate that IL-7 might significantly contribute to the local pro-inflammatory response observed in the salivary glands of patients with Sjögren's syndrome (SS). Therefore the purpose of our study was to study IL-7 expression in patients with primary SS (pSS) in relation to immunopathology and to investigate its potential immunoregulatory role in these patients.

**Methods:** Labial salivary gland (LSG) IL-7 expression was determined by immunohistochemistry using a quantitative scoring system in 30 sicca patients; 15 with primary (pSS) and 15 with non-Sjögren's sicca syndrome (nSS). LSG IL-7 expression was correlated to local and peripheral parameters of disease activity. IL-7 levels (ELISA) were also measured in the saliva and serum of pSS patients compared to healthy controls. Additionally, in vitro the effect of IL-7 on production of proinflammatory cytokines, chemokines and T-cell cytokines by peripheral blood mononuclear cells (PBMC) from pSS patients was determined by a Luminex multi-cytokine assay.

**Results:** The LSG IL-7 expression was significantly higher in pSS patients compared to nSS patients ( $p = 0.003$ ). IL-7 was primarily found in the vicinity of lymphocytic infiltrates and was produced by endothelial cells and a minority of CD68<sup>+</sup> macrophages. In addition, cells with fibroblast and myoepithelial cell morphology expressed IL-7. In saliva of pSS patients compared to healthy controls IL-7 levels were also significantly increased ( $p < 0.05$ ). Although serum IL-7 levels in pSS were slightly increased compared to nSS patients this was not statistically significant. In the pSS group as well as in the whole sicca patient group, LSG IL-7 scores significantly correlated (all  $p < 0.05$ ) with both local and peripheral disease parameters. IL-7 stimulated production of cytokines that contribute to activation of pro-inflammatory Th1 cells (IL-12 and IL-15) and induced Th1 cytokines (IFN $\gamma$ ) as well as chemokines that facilitate migration of Th1 cells (MIG and IP-10, all  $p < 0.05$ ). This was in contrast to IL-4, the major Th2 defining cytokine, which was not significantly changed by IL-7. IL-7 also significantly elevated IL-1 $\alpha$  and TNF $\alpha$ , proinflammatory cytokines that are able to induce immunopathology by their catabolic effects on tissue cells.

**Conclusions:** The correlation of LSG IL-7 expression with immunopathology and the immunostimulatory capacity of IL-7 indicate IL-7 to actively contribute to inflammation-induced pathology in patients with primary Sjögren's syndrome.

### 035 FOXP3<sup>+</sup> T REGULATORY CELLS (Tregs) IN THE AUTOIMMUNE LESIONS OF SJÖGREN'S SYNDROME (SS): CORRELATION WITH THE NUMBER OF INFILTRATING DENDRITIC CELLS AND MACROPHAGES

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Tregs are considered to participate in the modulation of autoimmune responses. The factors that mediate the differentiation

and/or accumulation of Tregs at the site of inflammation continue to be dissected and are thought to include a conducive cytokine milieu and favourable interactions with dendritic cells (DC). In SS, a chronic autoimmune exocrinopathy (mainly of the salivary and lacrimal glands) associated with glandular lymphocytic infiltrates, we have previously shown that FOXP3<sup>+</sup> Tregs are present in the minor salivary gland (MSG) inflammatory lesions and that their incidence correlates with the severity of the inflammatory infiltration.

**Purpose:** To investigate potential associations between the numbers of infiltrating Tregs and DC or other types of antigen-presenting cells (macrophages; MΦ) at the SS autoimmune lesions.

**Methods:** MSG biopsy specimens from 30 SS patients and 13 controls were studied by immunohistochemistry. SS samples were classified according to MSG lesion severity in three groups (10 patients each), consisting of samples with mild, intermediate or advanced MSG lesions (Tarpley biopsy score 1+, 2+ or 3/4+), respectively. Control group included 6 sicca-complaining subjects with negative biopsy scores that did not fulfil the SS American-European classification criteria and 7 with sialadenitis (3 sarcoidosis, 4 viral (3 HCV, 1 HIV)-related). Tregs, T cells, B cells, follicular DC, interdigitating DC and MΦ were identified by antibodies to FOXP3, CD3, CD20, fascin, S100 and CD68, respectively. Cells were counted field by field in each section. Statistical analyses were performed by Mann-Whitney and Spearman tests.

**Results:** FOXP3<sup>+</sup> Tregs were detected in all SS and sialadenitis-control samples, but not in the controls. The mean of the % FOXP3<sup>+</sup>/CD3<sup>+</sup> cells  $\pm$  SE was found  $7.54 \pm 1.13$  in SS and  $7.70 \pm 1.81$  in sialadenitis samples, whereas in the SS groups with mild, intermediate and advanced MSG lesions was  $2.24 \pm 0.50$ ,  $13.21 \pm 1.93$  and  $6.65 \pm 1.10$ , respectively. In both SS and sialadenitis-controls, FOXP3<sup>+</sup> cells were found to significantly correlate to the number of infiltrating mononuclear cells ( $r:0.90$ ,  $p<0.0001$  and  $r:0.96$ ,  $p<0.01$  in SS and controls, respectively), fascin<sup>+</sup> DC ( $r:0.90$ ,  $p<0.0001$  and  $r:0.93$ ,  $p<0.01$ ), S100<sup>+</sup> DC ( $r:0.53$ ,  $p<0.05$  and  $r:0.75$ ,  $p=0.07$ ) and CD68<sup>+</sup> MΦ ( $r:0.86$ ,  $p<0.0001$  and  $r:0.86$ ,  $p<0.05$ ), whereas they were negatively correlated to the CD3<sup>+</sup>/CD20<sup>+</sup> cell ratio ( $r:-0.65$ ,  $p=0.001$  and  $r:-0.79$ ,  $p<0.05$ ).

**Conclusions:** The comparable FOXP3<sup>+</sup> Treg incidence in the SS autoimmune and of other etiology inflammatory MSG lesions suggests that mechanisms related to Treg recruitment/induction are probably not impaired in SS. Although further investigation is needed to elucidate the factors mediating Treg accumulation at MSG infiltrates, DC and MΦ might be implicated.

### 036 T LYMPHOCYTE CLONAL ALTERATIONS IN ANTI-CITRULLINATED PROTEIN ANTIBODY POSITIVE SYNOVITIS

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**Background:** Increasing evidence suggests that the pathophysiology of rheumatoid arthritis (RA) is fundamentally different in patients with or without anti-citrullinated protein antibodies (ACPA). The association of RA with the HLA-DR shared epitope exclusively in the ACPA<sup>+</sup> subset supports this concept and suggests that T cell help may be involved in the ACPA response.

**Objectives:** We aimed to directly assess the potential involvement of T lymphocytes in ACPA<sup>+</sup> synovitis.

**Methods:** Synovial biopsies were obtained from actively inflamed knee joints of 54 RA patients. Of these, 37 were ACPA<sup>+</sup> as assessed by the anti-CCP2 ELISA. Synovial inflammation was assessed by standard synovial histology and immunohistochemistry. Total RNA was extracted from the biopsies of 11 ACPA<sup>+</sup> RA synovia, 7 ACPA<sup>-</sup> RA synovia, and 10 control spondyloarthritis (SpA) synovia with similar levels of T and B cell infiltration. T lymphocyte

clonality in the inflamed synovium was studied by combined quantitative and qualitative TCR CDR3 analysis by the TcLandscape technology. Alterations of the normal TCR length distribution were analyzed for all Vβ families, after correction for the number of specific transcripts and using Cβ as internal control.

**Results:** Comparing ACPA<sup>+</sup> with the ACPA<sup>-</sup> RA patients, there were no significant differences in demographic features, disease duration, treatment, and global disease activity. At the histological level, there was no difference in global inflammatory infiltration, synovial lining hyperplasia, vascularity, and infiltration with CD3<sup>+</sup> T cells, CD20<sup>+</sup> B cells, and CD138<sup>+</sup> plasma cells.

The TcLandscape technology combines a quantitative and qualitative TCR CDR3 analysis. This TCR analysis showed a significantly higher degree of alteration of the TCR length distribution (summarized for all Vβ families) in ACPA<sup>+</sup> synovitis ( $29.11 \pm 8.6\%$ ) compared to ACPA<sup>-</sup> synovitis ( $19.5 \pm 4.6\%$ ;  $p=0.011$ ). There was also a significant difference between ACPA<sup>+</sup> synovitis and SpA synovitis ( $22.6 \pm 4.2\%$ ;  $p=0.012$ ). However, the pool of all RA synovial tissue ( $23.66 \pm 7.8$ ) showed no difference compared to SpA synovitis. The differences in alteration were observed in several Vβ families and were different in each individual sample. These alterations reflecting oligo- or monoclonal expansions were confirmed by Jβeta analysis.

**Conclusion:** This study demonstrates an increased alteration of the TCR length distribution in ACPA<sup>+</sup> synovitis but not in ACPA<sup>-</sup> RA synovitis or control synovitis. These data suggest a specific contribution of T lymphocytes in the local disease process and possibly the ACPA response in this RA subset.

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### 037 IL-1 DRIVES PATHOGENIC TH17 CELLS DURING SPONTANEOUS ARTHRITIS IN IL-1RA-DEFICIENT MICE

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IL-1 receptor antagonist-deficient (IL-1Ra<sup>-/-</sup>) mice spontaneously develop an inflammatory and destructive T cell-driven arthritis due to unopposed excess IL-1 signalling. In this study, the role of Th17 and the effect of neutralizing IL-17 and IL-1 were investigated.

CD3<sup>+</sup> T cells were isolated from spleen and lymph nodes of IL-1Ra<sup>-/-</sup> mice and wild-type Balb/c mice. These T cells were stained for IL-17 and IFNγ and analysed by FACS. Compared to WT mice, IL-1Ra<sup>-/-</sup> mice had similar levels of IFNγ-positive Th1 cells, but showed clearly enhanced levels of IL-17-positive Th17 cells. Even young IL-1Ra<sup>-/-</sup> mice (6 weeks) without any macroscopic signs of arthritis already showed increased Th17 levels in both spleen and lymph nodes, while Th1 levels were unaffected. The percentage of IL-17-positive T cells increased even more after the onset of arthritis, and clearly correlated to the severity of arthritis.

In addition to these FACS analyses of spleen and lymph nodes, the sera of these mice were analysed for the expression of various cytokines using Luminex bead assays. IL-1, IL-17, and TNF were not (or hardly) detected in wild-type Balb/c sera, but these three cytokines were clearly expressed in the serum of IL-1Ra<sup>-/-</sup> mice, and 2the levels correlated significantly to the age and severity of arthritis.

To investigate the contribution of IL-1 and IL-17 in the further progression of the disease, these cytokines were blocked with neutralizing antibodies after onset of arthritis. Anti-IL-17 treatment compared to isotype control prevented a further increase in inflammation and bone erosion. In contrast, blocking of TNF after onset of arthritis had no effect. Neutralizing IL-1 on the other hand resulted in a complete suppression of the arthritis. Interestingly,

this anti-IL-1 treatment also significantly reduced the percentage of IL-17-positive Th17 cells in the draining lymph nodes.

This study comparing wild-type Balb/c mice with naïve and arthritic IL-1Ra<sup>-/-</sup> mice shows that an increase of Th17 already precedes the onset of arthritis in IL-1Ra<sup>-/-</sup> mice. In addition, our blocking studies demonstrated that IL-17 contributes to the inflammation and bone erosion in this model, and suggest that IL-1 is the driving force behind the IL-17-producing Th17 cells.

### 038 PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCs) OF SYSTEMIC SCLEROSIS PATIENTS INDUCE APOPTOSIS IN CO-CULTURED AUTOLOGOUS FIBROBLASTS AND UP-REGULATE HLA-DR AND FASL

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**Purpose:** In the last years, several data suggested that an interplay between T cells and fibroblasts plays a pivotal role in promoting matrix accumulation in systemic sclerosis (SSc). We have recently shown that peripheral blood T cells of SSc patients co-cultured with autologous fibroblasts expand the same T cell clonotypes that are found to be increased in the affected skin. The aim of this work was to analyze functional changes in co-cultured cells, to provide evidence of a further pathway in the cross-talk between specific immune system cells and fibroblasts in SSc.

**Methods:** Peripheral blood mononuclear cells (PBMCs) and skin fibroblasts were obtained from SSc patients undergoing biopsy for diagnostic purposes. Cells were co-cultured at 37°C 5% CO<sub>2</sub> for 10 days in complete medium supplemented with r-IL2. Then cells were stained with monoclonal antibodies for CD3, CD4, TCRαβ, TCRγδ, HLA-DR, FASL, AnnexinV, FAS and analyzed by flow-cytometry using a FACScalibur.

**Results:** In SSc autologous T lymphocytes-fibroblasts co-cultures, T cells bearing an αβ receptor were expanded and were positive for the expression of HLA-DR, suggesting their activation. Moreover we found an up-regulation of FASL on T CD4<sup>+</sup> cells, consistent with the up-regulation of FAS on autologous SSc fibroblasts and their progressive increase in apoptosis.

**Conclusion:** The antigenic activity of scleroderma fibroblasts on autologous T cells is, once again, strongly supported. In addition, the association of the FAS/FASL pathway with fibroblasts apoptosis in co-cultures with autologous T cells may suggest an important role of this mechanism in SSc pathogenesis, as we already suggested in previous experiments.

### 039 RENAL INFLAMMATORY INFILTRATE IN LUPUS NEPHRITIS PATIENTS

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**Background:** Pathogenesis and prognostic factors of lupus nephritis are still not completely known. Recent studies have revealed the implication of lymphocytic interstitial infiltrate in the pathogenesis and prognosis of systemic lupus erythematosus (SLE) nephritis.

**Objective:** To evaluate the presence, distribution and phenotype of the inflammatory infiltrate in renal tissue of SLE nephritis patients and possible correlations with clinical or histological parameters and response to therapy.

**Methods:** 31 consecutive patients with newly diagnosed lupus nephritis were included in the study. Demographic, clinical, immunological and renal evolution data were collected. Renal biopsies were classified according to the WHO criteria and examined for activity and chronicity indexes. In addition to routine staining, the tissues were stained by immunohistochemistry

using as cellular markers CD3 (T lymphocytes), CD20 (B lymphocytes) and CD68 (macrophages). Cellular count was performed for each phenotype and results were expressed as number of positive cells on total biopsy surface. The infiltrate was considered as present if the number of total infiltrating lymphocytes was >10 cells.

At 6th month of immunosuppressant therapy, patients were considered responder or non-responder on the basis of 24 hour proteinuria <1 g and creatinine levels reduction.

**Results:** Characteristics of the 31 patients are 27 women, 4 men; age at renal biopsy 35.6±11.6; number of class IV WHO = 31; activity index 9.1±4.3 and chronicity index 2.1±1.9. The number of CD3<sup>+</sup>, CD20<sup>+</sup> and CD68<sup>+</sup> cells were 17.7±23.4, 13.1±25.4 and 47.1±41.7, respectively. The leukocyte infiltrate was prevalently interstitial, without specific sublocalization.

A direct correlation between chronicity index and presence of CD3 (r = 0.40, p 0.025) and CD20 (r = 0.59, p 0.001) positive cells was found. No correlations were observed with other clinical, laboratory or histological parameters. Thirteen out of 14 patients without infiltrate were responder with respect to 10/17 with infiltrate (93% vs 59%, p = 0.045). The baseline creatinine values (1.2±0.5 vs 1.0±0.7; p = 0.03) and the chronicity index (2.8±2.2 vs 1.2±1.0; p = 0.04) were higher in the second group.

The 23 responder patients differ from 8 non-responder only for the presence of inflammatory infiltrate (43.5% vs 87.5%, p = 0.045) and the number of CD3<sup>+</sup> cells (13.7±22.3 vs 29.4±24.0, p = 0.03).

On multivariate analysis, the inflammatory infiltrate resulted in being the independent predictive parameter of response, even if with a borderline statistical significance (p = 0.05, OR 9.1 95% IC 1.0–86.1).

**Conclusions:** These data support the hypothesis that cell-mediated immunity plays a central role in the SLE nephritis pathogenesis. In particular, the higher values of creatinine and chronicity index observed in patients with infiltrate suggest this possible function in the progression of nephritis up to chronic damage.

### 040 SIZE OF CD8 T CELL COMPARTMENT IN SYNOVIAL FLUID PREDICTS EXTENSION IN CHILDREN WITH OLIGOARTICULAR JUVENILE IDIOPATHIC ARTHRITIS

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**Background:** Extended oligoarticular juvenile idiopathic arthritis (JIA) is a chronic debilitating form of childhood arthritis that arises unexpectedly from an initially mild onset. We have examined the cellular and molecular composition of the first synovial fluid (SF) aspirates from JIA patients with up to four affected joints to discover markers that will predict extension from a mild phenotype (called persistent oligoarticular JIA) to the more severe phenotype (extended oligoarticular JIA).

**Methods:** Flow cytometry was used to quantify inflammatory and regulatory subsets of T cells, NK cells, B cells, dendritic cells, macrophages and other CD13<sup>+</sup> cells in 27 (18 persistent outcome, 9 extended outcome) synovial fluid mononuclear cell (SFMC) samples. From this group, 11 (5 persistent outcome, 6 extended outcome) SF RNA samples were hybridized to Affymetrix U133v2 gene expression microarrays.

**Results:** Although there was a trend towards diminished numbers of CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells in the SF of patients whose disease later extended, the most significant difference was the predominance of CD8<sup>+</sup> cells in the T cell compartment of these patients (mean CD3<sup>+</sup>CD8<sup>+</sup> = 37.9±8.4% of live cells in children whose disease would remain persistent oligoarticular versus 46.0±11.4% in those whose disease phenotype would extend p = 0.05). Seventy six genes were found to be differently expressed between the two phenotypic groups at a significance level p<0.001. Selected



candidate genes are being tested for their predictive value on new first SF samples.

**Conclusions:** Our data suggest that in children with early arthritis, future worsening of disease can be predicted, either by cell composition or gene expression, from cells within the synovial fluid. If these findings are replicated in a new set of samples, they may represent a major step forward in our understanding of mechanisms that underlie severity in childhood arthritis.

#### 041 HIGH PROPORTION OF CD4+ CD25<sup>high</sup> REGULATORY T-CELLS IN REMISSION OR LOW DISEASE ACTIVITY RHEUMATOID ARTHRITIS

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**Objectives:** Our aim was to quantify the proportion of CD4+ CD25<sup>high</sup> regulatory T-cells and TGF- $\beta$  mRNA expression in the peripheral blood of remission or low disease activity rheumatoid arthritis (RA) patients.

**Methods:** Peripheral blood samples from patients with remission or low disease activity RA (n = 15) and healthy controls (n = 8) were examined. CD4+ CD25<sup>high</sup> T-cells and CD4+ CD25- T-cells were quantified using flow cytometry. TGF- $\beta$  mRNA expression by peripheral blood leucocytes was quantified using real time quantitative PCR.

**Results:** There was a higher proportion of CD4+ CD25<sup>high</sup> T-cells and a smaller proportion of CD4+ CD25- T-cells in the peripheral blood of remission or low disease activity RA patients than in healthy controls. There was a negative association between C-reactive protein values and the proportion of CD4+ CD25<sup>high</sup> T-cells. There were no differences in TGF- $\beta$  mRNA expression between groups.

**Conclusions:** These data suggest that CD4+ CD25<sup>high</sup> regulatory T-cells may have an important role in inducing or maintaining remission. The reinforcement of the number and competence of these cells may be a useful therapeutic goal in RA treatment research.

## Immune regulation, cellular interactions and molecular pathways

### Oral presentation

#### 042 EVIDENCE FOR ALTERATIONS IN INNATE IMMUNITY IN THE PRECLINICAL PHASE OF RHEUMATOID ARTHRITIS

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**Background:** Rheumatoid arthritis (RA) is a destructive inflammatory disease affecting mostly the joints. To prevent irreversible joint damage, early diagnosis and start of treatment is of high importance. Individuals that are positive for anti-citrullinated protein/peptide antibodies (ACPA) and/or rheumatoid factor (RF) are at high risk for developing RA.

**Objective:** The identification of changes in the immune system in the preclinical phase of disease to understand the etiology of disease and to identify additional biomarkers to improve predictive power with as the ultimate goal preventive medicine.

**Methods:** Individuals (n = 107) positive for ACPA and/or RF were recruited to the clinic and their physical health was followed for several months (range 7.83–37.97 months). During this follow-up

time 16 individuals were diagnosed for arthritis. Gene expression profiles from peripheral blood cells were analyzed for 19 persons at risk and compared with the profiles of 6 control persons who were ACPA and RF negative. Significance Analysis of Microarrays and hierarchical clustering was used to analyze the data. To interpret the results we used pathway-level analysis. The expression of selected target genes was validated in the total group of 107 persons at risk using Taqman Low Density Arrays (TLDA) and compared to a group of 23 controls and 24 RA patients with established disease.

**Results:** Large scale gene expression analysis between individuals at risk for developing RA and healthy individuals revealed remarkable significant differences in gene expression profiles. Among these differences we observed expression level changes in genes involved in the innate immunity, which was especially prominent in a subgroup of individuals at risk. Expression levels of selected genes were confirmed using the TLDA method in the total cohort of patients at risk for RA and compared to levels in established RA patients and controls negative for ACPA and RF. Interim analyses based on persons at risk with at least 24 months clinical follow-up (n = 46) identified potential predictive biomarkers for the development of RA.

**Conclusions:** This study shows that persons at risk for RA display a highly distinct gene expression profile reflecting ongoing changes in the innate immune system in the preclinical phase of RA. Longer follow-up is expected to yield additional biomarkers and increased predictive power.

### Oral presentation

#### 043 T AND B CELL INFILTRATION IN THE GASTRIC MUCOSA OF SYSTEMIC SCLEROSIS PATIENTS

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**Objective:** Systemic sclerosis (SSc) is a connective tissue disorder characterised by microvascular changes and immunological abnormalities leading to progressive widespread fibrosis of the skin and visceral organs. We have recently shown that a severe fibrosis and increased expression of several profibrotic cytokines are important hallmarks in the gastric wall of SSc patients.<sup>1</sup> The present study was performed to analyse whether microvascular alterations and/or inflammation might contribute to the fibrotic response in gastric tissue from SSc patients.

**Methods:** Gastric biopsy samples were obtained during esophago-gastroscopy, from the corpus and antrum of 20 SSc patients and 10 healthy controls. Skin biopsy samples were obtained from the involved skin of 3 SSc patients and from 3 controls. Samples were immediately snap-frozen and serial tissue sections were immunostained for CD45 (LCA), CD3 (T cells), CD4 (helper T cells), CD8 (cytotoxic T cells), CD20 (mature B cells), CD68 (macrophages), very late antigen-4 (VLA-4), vascular cell adhesion molecule-1 (VCAM-1), the endothelial-specific marker CD31 (PECAM-1) and vascular endothelial growth factor (VEGF). Microvascular density in the specimens was assessed by counting the number of CD31-positive vessels/high-power field (X20) by two independent observers blinded with regard to the biopsy classification.

**Results:** A massive T cell infiltration in the lamina propria was a prominent finding in many of the specimens from SSc patients. CD4+ and CD8+ T cells could be detected with helper T cells being predominant in the tissue. T cells were found in both lymphocyte aggregates and diffuse lymphocyte infiltrates and strongly expressed the activation marker VLA-4. Mature B cells were frequently found arranged in clusters and were rarely seen in a

diffuse pattern. Most lymphocyte aggregates lacked macrophages that were observed in the lamina propria of SSc gastric mucosa in some samples. VCAM-1 was detected in some SSc specimens, restricted to areas with massive perivascular inflammatory infiltrates. No difference in microvascular density was observed between SSc and controls. No expression of VEGF could be detected in gastric tissue from SSc patients, while a strong expression was found in SSc involved skin.

**Conclusion:** Our data suggest that similar to the findings in SSc skin, activated T and B cell infiltration may contribute to the fibrotic response and tissue damage within the gastric mucosa of SSc patients, without evidence of defective angiogenesis. This study supports the importance of immunohistochemical examination on gastroscopy samples that could be useful for detecting early stages and/or different subtypes of gastrointestinal disease in SSc.

This study was in part supported by a grant of the German Network for Systemic Sclerosis (DNSS).

1. **Manetti M, et al.** Severe fibrosis and increased expression of fibrogenic cytokines in the gastric wall of systemic sclerosis patients. *Arthritis Rheum* 2007;**56**:3442–7.

## Oral presentation

### 044 ACTIVITY-ASSOCIATED UPREGULATION OF TNF AND IL-18 IN GLOMERULI AND SERA OF SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS

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**Objective:** To investigate IL-18 and TNF renal expression and plasma levels, their association with disease activity, and the effects of therapeutic TNF blockade on IL-18 in systemic lupus erythematosus (SLE) patients.

**Methods:** Renal biopsies of 15 SLE patients with glomerulonephritis (1 class I, 4 class II, 4 class III, 3 class IV, 3 class V) were graded for nephritis activity and analyzed by immunohistochemistry for membrane-bound TNF and for IL-18. Independent examiners estimated expression in a semi-quantitative way. Serum levels of IL-18 of 40 patients fulfilling ACR criteria for SLE and of 20 healthy individuals (HC) were determined by ELISA. TNF was measured by ELISA in 35 of these patient samples and 20 HC. Disease activity was measured by SLE index score (SIS). Six SLE patients were treated with four infusions of the humanized chimeric anti-TNF antibody infliximab at time 0 and after 2, 6, and 10 weeks in combination with azathioprine or methotrexate in an open label safety and feasibility study approved by the local ethics committee, and IL-18 serum levels were determined by ELISA.

**Results:** Glomeruli of patients with lupus nephritis contained significant amounts both of IL-18 and locally produced (membrane-bound) TNF. Nephritis activity correlated with TNF expression ( $r = 0.58$ ,  $p < 0.05$ ) and showed a trend towards correlating with IL-18 expression ( $r = 0.46$ ,  $p = 0.088$ ). As compared to HC sera, SLE sera contained increased TNF ( $63 \pm 44$  pg/ml (mean  $\pm$  SD) vs  $15 \pm 4$  pg/ml,  $p < 0.0001$ ) and IL-18 ( $91 \pm 44$  pg/ml vs  $36 \pm 12$  pg/ml,  $p < 0.0001$ ). SLE disease activity as measured by SIS correlated with TNF ( $r = 0.76$ ,  $p < 0.0001$ ), and, to a lesser degree, with IL-18 ( $r = 0.38$ ,  $p < 0.02$ ). IL-18 was highly correlated with TNF ( $r = 0.59$ ,  $p = 0.0002$ ). Under therapeutic TNF blockade with infliximab, IL-18 fell from  $54 \pm 24$  pg/ml to a minimum of  $26 \pm 14$  pg/ml after six weeks ( $p < 0.05$ ), but increased again to  $62 \pm 63$  pg/ml 10 weeks after anti-TNF therapy was stopped.

**Conclusions:** Both TNF and IL-18 are highly increased in active SLE, in the inflamed organs as well as in sera. TNF appears more closely linked to SLE disease activity, while the overexpression of

IL-18, at least in part, is secondary to TNF upregulation. IL-18 overexpression therefore may constitute a major pathogenic consequence of high TNF levels in SLE.

## Oral presentation

### 045 IL-33 REDUCES THE DEVELOPMENT OF ATHEROSCLEROSIS

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Cardiovascular disease is a leading cause of mortality in rheumatoid arthritis and many immunological and metabolic factors may be involved. Our group has previously shown that soluble ST2 was therapeutic in a murine model of collagen-induced arthritis (CIA). Here, we show that IL-33, a novel IL-1-like cytokine that signals via the receptor ST2, can reduce atherosclerosis development in ApoE<sup>-/-</sup> mice on high fat diet, while soluble ST2 treatment increased plaque size. IL-33 and ST2 are both present in the normal and atherosclerotic vasculature of mouse and humans. While control PBS-treated mice developed severe and inflamed atherosclerotic plaques in the aortic sinus, lesion development was profoundly reduced in IL-33-treated animals (intimal plaque area  $0.42 \pm 0.04$  vs  $0.16 \pm 0.02$  mm<sup>2</sup>,  $P < 0.0001$ ). IL-33 also markedly increased levels of IL-4, IL-5, and IL-13 but decreased levels of IFN $\gamma$  in serum and lymph node cells. IL-33-treatment also elevated levels of total serum IgA, IgE and IgG1 but decreased IgG2a, consistent with a Th1-to-Th2 switch. IL-33-treated mice also produced significantly elevated anti-ox-LDL antibodies. Conversely, mice treated with soluble ST2, a decoy receptor which neutralizes IL-33, developed significantly larger atherosclerotic plaques in the aortic sinus of the ApoE<sup>-/-</sup> mice compared to control IgG-treated mice (intimal plaque area  $0.66 \pm 0.05$  vs  $0.35 \pm 0.05$  mm<sup>2</sup>,  $p = 0.0015$ ). Furthermore, co-administration of an anti-IL-5 mAb with IL-33 prevented the reduction in plaque size and reduced the amount of ox-LDL antibodies induced by IL-33. In conclusion, IL-33 may play a protective role in the development of atherosclerosis via the induction of IL-5 and ox-LDL antibodies, whereas soluble ST2 increased atherosclerosis. The effect of novel biologic agents targeting rheumatoid arthritis should also be examined in the context of atherosclerosis, since cytokine effector function is context dependent and may mediate discrete function across different tissues within one autoimmune disorder.

## Oral presentation

### 046 TRAIL-INDUCED RA SYNOVIAL FIBROBLAST CELL DEATH IS MEDIATED BY CASPASES AND REGULATED BY THE PI3K/AKT PATHWAY

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**Rational:** A hallmark of rheumatoid arthritis (RA) is the pseudo-tumoral expansion of fibroblast-like synoviocytes (FLS), and RAFLS have been proposed as a therapeutic target. TNF-related apoptosis-inducing ligand (TRAIL) has been described as a pro-apoptotic factor on RAFLS and suggested as a potential drug. We have previously showed that exposure to TRAIL induces apoptosis only in a portion of RAFLS. In the surviving cells, TRAIL induced RAFLS proliferation, complicating the proposed strategy to use TRAIL in the treatment of RA. To evaluate possibilities to overcome TRAIL resistance in RAFLS, we study the intracellular pathways implicated in TRAIL-induced signalling.

**Material and methods:** To examine the implication of caspases in TRAIL apoptosis, RAFLS were stimulated with TRAIL and cleavage was assessed by western blot. p21 and p27 expression were examined by western blot. We evaluated the participation of

caspase-activation, but also ERK1/, p38 MAP Kinases and Akt in the regulation of TRAIL-induced apoptosis and proliferation. RAFLS were pre-treated for one hour with specific chemical inhibitors and cultured with TRAIL for 24 hours. Apoptosis was then analyzed by flow cytometry on using annexin V-FITC binding and TOPRO-3 up-take. Proliferation was measured using incorporation of [3H] thymidine.

**Results:** Inhibition of MAP or PI3 kinases decreased significantly TRAIL-induced proliferation of RAFLS, but only Akt inhibitors also increased TRAIL-induced cell death. Caspases 8, 3 and 9 are activated after TRAIL treatment and their inhibitions reduced TRAIL-induced cell death. A pan-caspases inhibitor (z-VAD-FMK) almost completely blocked TRAIL-induced apoptosis, but also blocked TRAIL-induced proliferation. Using specific inhibitors for caspases 8, 3 and 9, we found that caspases 8 and 3 may be involved in both apoptosis and proliferation induced by TRAIL, while caspase 9 is only involved in TRAIL induced apoptosis. Because MAPK and Akt pathways are involved in TRAIL induced proliferation, we tested whether caspases participate on MAPK and Akt pathways activation after TRAIL treatment. This hypothesis was not confirmed, since inhibition with z-VAD-FMK did not have any effect on TRAIL-induced MAP and PI3 kinases activation. We analyzed expression of p21 and p27, two inhibitory proteins of the cell cycle progression. p21 and p27 are cleaved after TRAIL treatment and this is blocked by addition of pan caspases inhibitor.

**Conclusion:** Inhibition of PI3 kinase/Akt signalling pathway abrogates TRAIL-mediated RAFLS proliferation and increases RAFLS susceptibility to TRAIL-induced apoptosis. TRAIL-induced apoptosis of RAFLS is mediated by the initiator caspase 8, the effector caspase 3 and also by the mitochondrial amplification loop of apoptosis through caspase 9 activation. Surprisingly is the observation that caspases are also participating in TRAIL-induced proliferation, possibly through the cleavage of key proteins in cell cycle regulation.

#### 047 LIVER X RECEPTORS PRESENT A NOVEL INFLAMMATORY PATHWAY IN RHEUMATOID ARTHRITIS

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Rheumatoid arthritis is a chronic inflammatory condition of unknown etiology. It is characterised by synovial inflammation, cell proliferation and cartilage and bone destruction. Many pro-inflammatory cytokines including TNF $\alpha$ , IL-1 $\beta$ , IL-6 and IL-17 have been implicated. Recently, we have studied a nuclear receptor, Liver X receptor, which we have explored as a potential upstream driver of synovial pathology. Liver X receptors (LXRs) belong to the superfamily of nuclear receptor ligand activated transcription factors and have been characterised mainly regarding lipid metabolism. LXRs are expressed in synovial tissue and immune cells including CD4+ and CD8+ T cells, dendritic cells, monocytes and macrophages. Administration of the LXR agonists, T1317 and GW3965 to LPS treated human CD14+ monocytes exacerbated release of multiple pro-inflammatory cytokines including IL-1, IL-6, IL-17 and TNF $\alpha$ . Secondly, in an in vitro model of joint inflammation, co-culture of cytokine activated T cells and MCSF treated monocytes, from both healthy and RA patients, in the presence of LXR agonists exacerbated TNF $\alpha$  secretion together with multiple other cytokines and chemokines. Furthermore, we demonstrated in an in vivo model of collagen-induced arthritis mice that LXR agonists induced higher incidence and severity of disease assessed both clinically and histologically. FACS analysis of intracellular cytokines in lymph node cells showed that LXR agonist treated mice had a higher percentage of IL-17 and IFN $\gamma$  positive CD4+ T cells. Serum cytokine analysis revealed upregulation of the cytokines IL-1 $\alpha$ , IL-10, IL-12, IL-17, GM-CSF and the chemokines

IL-8, MIP-1 $\alpha$  and MIG. We conclude that LXR agonism is pro-inflammatory in vitro and in vivo and that the LXR pro-inflammatory pathway may represent a novel inflammatory pathway that may initiate or sustain inflammation in rheumatoid arthritis.

#### 048 TRANSFORMING GROWTH FACTOR-BETA ACTIVATED KINASE 1 (TAK1) IS A CENTRAL MEDIATOR IN RHEUMATOID ARTHRITIS

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**Introduction:** Rheumatoid arthritis (RA) is the most frequent rheumatic condition characterized by chronic inflammation within the joint tissue. While current biotherapies neutralizing TNF have revolutionized the treatment of RA, they still have drawbacks supporting the need for the development of alternative therapeutic approaches. Transforming growth factor-beta activated kinase 1 (TAK1), a downstream mediator of IL-1 and TNF signal pathways, plays an important and central role in the regulation of catabolic events and inflammatory processes in the context of degenerative joint diseases like RA. TAK1 is thus an attractive drug target in attempts to reduce both joint inflammation and degradation. We propose to investigate the feasibility of targeting the TAK1-mediated inflammatory cascades by RNA interference (RNAi) in an experimental model of arthritis using innovative therapeutic approaches.

**Methods:** TAK1 siRNA sequences were validated in vitro with 10T1/2 and C2C12 mesenchymal progenitor cells as well as the macrophage-cell line J774.1, assessing the protein levels by western blotting and by downregulation of p38- and JNK-activation after stimulation with cytokines. For in vivo administration, 10  $\mu$ g of siRNA were formulated as lipoplexes with the RPR209120/DOPE liposome and a carrier DNA, and injected intravenously in DBA/1 mice having collagen-induced arthritis (CIA). Clinical course of the disease was assessed by paw thickness over time, and radiological and histological scores were obtained at euthanasia. The cytokine profiles were measured by ELISA in sera and knee-conditioned media. The immunological balance was assessed using anti-type II collagen (bCII) assays and measuring the bCII-specific T cell proliferation. The cellular mechanisms of lipoplex action were investigated using fluorescent siRNA (siGLO).

**Results:** The TAK1 siRNA sequence reproducibly silenced at least 50% of the protein expression compared with a control siRNA. In the CIA model, weekly intravenous injections of anti-TAK1 siRNA-lipoplexes significantly reduced incidence and severity of arthritis, abrogating joint swelling, in established arthritis compared with the control siRNA lipoplex-injected group. The siRNA formulation was widely distributed, delivering the siRNA to several organs with a strong efficacy in liver and spleen. The molecular and cellular mechanisms responsible for such a striking therapeutic effect are currently under investigation.

**Conclusion:** TAK1 being implicated in pathways regulating inflammation, synovial proliferation and bone homeostasis, it appears as an attractive target able to reduce inflammation and suppress structural changes in RA.

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#### 049 TPL2 KINASE REGULATES Fc $\gamma$ R AND TLR-INDUCED ERK1/2 ACTIVATION AND CYTOKINE PRODUCTION IN PRIMARY MURINE AND HUMAN MACROPHAGES

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**Background:** Accumulated evidence supports the role of Fc $\gamma$ Rs and TLRs in the regulation of autoimmune and inflammatory diseases.



Signalling pathways activated downstream of FcγRs and TLRs include, among others, the MAPK and NF-κB pathways that are involved in macrophage activation, differentiation and survival. Tpl2 is a ser/thr kinase of the MAP3K family that preferentially regulates activation of ERK1/2 MAPKs and pro-inflammatory cytokine production in response to LPS. This is supported by data whereby Tpl2 deficient mice are resistant to LPS induced septic shock and inflammatory bowel disease.

**Objectives:** We evaluated the role of Tpl2 kinase in the activation of MAPK and NF-κB signalling pathways and gene expression after cross-linking the FcγRs and TLRs in primary murine macrophages and human monocytes.

**Methods:** Bone marrow derived macrophages were differentiated from wild type C57BL/6 and Tpl2<sup>-/-</sup> mice by using rmMCSF. Thioglycolate elicited murine peritoneal macrophages were selected by plastic adhesion. Human monocytes were purified from peripheral blood mononuclear cells by magnetic beads. FcγRs were cross-linked by using plate bound IgG and Pam3Cys4, Zymosan, poly dI:dC, LPS and CpG oligonucleotides were used as specific TLR ligands. Whole cell extracts were analyzed by using Western blots and specific antibodies and gene expression and cytokine production by using real time PCR and specific ELISA assays.

**Results:** Cross-linking of the FcγRs and stimulation with Pam3Cys, polyI:C and LPS inhibited phosphorylation of ERK1/2 MAPK in Tpl2 deficient macrophages. However, phosphorylation of p38 and JNK MAPKs, as well as NF-κB activation was not significantly affected. Inhibition of ERK1/2 activation in Tpl2 deficient macrophages correlated with decreased expression of TNF-α after LPS and IgG stimulation; in contrast, the expression of the chemokine genes RANTES, MCP-1 and COX-2 was not significantly affected. Moreover, Tpl2 deficiency inhibits FcγR-, TLR2-, TLR3- and TLR4- induced TNF-α, IP-10 and IL-10 production and the downstream STAT activation. However, Tpl2 deficiency did not affect FcγR expression and phagocytosis in macrophages. Finally, pharmacologic inhibition of Tpl2 in primary human macrophages led to specific inhibition of ERK1/2 phosphorylation after FcγRs cross-linking and stimulation with TLR ligands.

**Conclusions:** Tpl2 kinase is required for ERK1/2 MAPK activation after FcγRs crosslinking and TLR stimulation in primary macrophages. Moreover, Tpl2 regulates FcγR-, TLR2-, TLR3- and TLR4-induced expression of downstream target genes and cytokine production. Deficiency or pharmacological inhibition of Tpl2 kinase leads to preferential blockade of production of the pro-inflammatory genes TNF-α and IP-10 and of the pleiotropic cytokine IL-10 and thus could represent a potential therapeutic target in systemic inflammatory and autoimmune diseases.

#### 050 TPL2 KINASE REGULATES THE INTEGRIN-INDUCED EFFECTOR FUNCTIONS IN PRIMARY MACROPHAGES: IMPLICATIONS FOR THE INFLAMMATORY RESPONSE

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**Background:** Integrin function is crucial for the function of neutrophils and macrophages, key effector cells during the innate immune response. Integrin signalling is critical for cellular functions such as firm adhesion, cell spreading, chemotaxis and the production of various cytokines. Thus, tight control of integrin signalling and function is required for appropriate coordination of innate immune and inflammatory responses. Signalling pathways activated downstream of integrins include the p38, ERK1/2 and JNK MAPKs among others. Tpl2 is a MAP3K that has been shown to preferentially regulate activation of ERK1/2 MAPKs and pro-inflammatory cytokine production in response to LPS.

**Objectives:** We sought to evaluate the role of Tpl2 kinase in the integrin signalling pathway and macrophage functions such as adhesion, spreading and cytokine production after integrin stimulation.

**Methods:** Bone marrow derived macrophages (BMDMs) and thioglycolate elicited peritoneal macrophages, were isolated from wild type C57/B6 and Tpl2 deficient mice. Moreover, CD14<sup>+</sup> monocytes were isolated from healthy individuals by magnetic resolution. Macrophages were plated on RGD coated surfaces to crosslink β1, β2 and β3 integrins. Whole cell extracts were analyzed by using western blots and specific antibodies and gene expression and cytokine production, by using real time PCR, flow cytometry and specific ELISA assays. Cell adhesion and spreading were analyzed after plating macrophages on surfaces coated with the specific integrin ligands fibronectin, collagen I, laminin and fibrinogen.

**Results:** Tpl2 deficiency affected cell spreading as assessed by contrast phase microscopy. Flow cytometry shown equal levels of integrin cell membrane expression in wt and Tpl2 deficient macrophages. Integrin activation on macrophages by the synthetic ligand RGD led to the phosphorylation of p38, ERK1/2 and JNK1/2 MAPKs in wt macrophages. Phosphorylation of ERK1/2 MAPK—but not of p38 and JNK1/2 MAPKs—was completely inhibited in Tpl2 deficient macrophages. The inhibition of ERK1/2 kinase activation in Tpl2 deficient macrophages was accompanied by significant inhibition of cytokine production such as TNF-α, IL-10 and IP-10. Moreover, decreased integrin-induced IL-10 production was accompanied by decreased STAT3 phosphorylation and STAT3-dependent gene induction in Tpl2 deficient cells. Finally, M-CSF-induced ERK1/2 phosphorylation and activation of NF-κB was not affected after integrin stimulation in wt and Tpl2 deficient macrophages.

**Conclusions:** Tpl2 kinase is required for integrin-induced ERK1/2 MAPK activation, cell spreading and cytokine and chemokine production in primary macrophages. Our data identify Tpl2 as part of the integrin signalosome that is formed in adherent macrophages. Tpl2 may represent a potential therapeutic target in systemic inflammatory/autoimmune diseases where aberrant integrin function may play a pathogenic role.

#### 051 INVESTIGATION OF THE EFFECTS OF INFlixIMAB (REMICADE) ON PERIPHERAL BLOOD CELLS OF RHEUMATOID ARTHRITIS PATIENTS 2 HOURS AFTER INFUSION

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**Background:** Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown aetiology that affects up to 1% of the adult population, and can be associated with severe damage to synovial joints and articular structures. TNF cytokine plays a key role in driving the pathogenesis and persistence of RA and blockade of TNF using current biological response modifiers has had profound therapeutic effects, with marked clinical improvement and radiographic benefit in a significant number of patients. However, the mechanisms of action of infliximab and the speed with which it exerts its effects are still unclear. This study aimed to establish the effect of infliximab infusion on numbers and phenotype of circulating peripheral blood leukocytes in RA patients.

**Methods:** All patients (n = 9) in this study have failed at least two DMARDs, and fulfil the revised 1987 ACR criteria for RA. Peripheral blood was obtained from patients, both pre- and 2 hours post-baseline infliximab infusion. Red cell lysis was performed on blood and leukocytes were stained for phenotypic markers, namely CD3, CD4 and CD8 for T cell populations, CD14 and CD16 for monocyte populations, CD19 for B cells and CD56 for NK cells and analysed using flow cytometry.

**Results:** Phenotypic profiling of peripheral blood leukocytes pre- and 2 hours post-infliximab infusion show that certain cell populations are specifically and rapidly affected by TNF blockade. There is a significant decrease of total CD14<sup>+</sup> monocyte counts 2 hours post infusion (p = 0.026). When monocytes were classified

by levels of CD14 expression a drop in the CD14<sup>high</sup> population was found to be mostly responsible for this decrease ( $p = 0.021$ ); interestingly, the CD14<sup>low</sup> population significantly increases in number within 2 hours of infliximab infusion ( $p = 0.028$ ); this population, however, is 10-fold less abundant than the CD14<sup>high</sup> monocytes. CD8 T cells and NK cells also increased significantly ( $p = 0.004$  and  $p = 0.017$ ).

**Conclusions:** Infliximab infusion caused significant changes in circulating monocyte levels within 2 hours of a baseline infusion; the CD14<sup>high</sup> monocytes were primarily targeted for depletion. This monocyte population is not thought to be a highly activated subset, and usually expresses low levels of membrane TNF; therefore the mechanism by which infliximab is targeting these cells is unclear, as is the way these cells are cleared from the blood. In vitro experiments are now underway to determine the mechanism of depletion and the pro-inflammatory potential of this CD14<sup>high</sup> population of monocytes. Demargination of leukocytes could explain the increase in other leukocyte populations. Studies are in progress on more infliximab treated RA patients, as well as comparison studies with other biological response modifiers, such as adalimumab and etanercept. The patients will also be stratified according to their response status, to examine whether this CD14<sup>high</sup> monocyte drop is related to the effectiveness of anti-TNF therapies.

## 052 ADOPTIVE TRANSFER OF CD4<sup>+</sup>CD49b<sup>+</sup> REGULATORY T CELLS EXPANDED BY IMMATURE DENDRITIC CELLS REVERSE SYMPTOMS OF ESTABLISHED ARTHRITIS

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**Objective:** We previously demonstrated that immature DCs (iDCs) prevent arthritis by inducing a regulatory population of T lymphocytes (Treg). These Tregs were characterized by the expression of the CD49b molecule and by interleukin-10 secretion. In the present study we evaluated and compared the therapeutic potential of iDCs versus Tregs on established arthritis. In order to better define the regulatory properties of these Tregs, we tested their anti-inflammatory potential in the oxazolone model of delayed type hypersensitivity (DTH) and we currently investigate their homing capacities to inflamed tissues.

**Methods:** Tregs were purified from the liver and spleen of DBA/1 mice that were repetitively injected with iDCs. These Tregs were adoptively transferred in DBA/1 mice with established CIA (day 28). In parallel repetitive iDCs injections were performed intraperitoneally in mice at various time points after the boost in order to compare their therapeutic potential to Tregs. For homing experiments, we plan to use the Thy1.1/Thy1.2 model in a Balb/C background in order to analyse the biodistribution of CD4<sup>+</sup>CD49b<sup>+</sup>Thy1.1<sup>+</sup> cells by flow cytometry 2 days after intravenous injection and local inflammation. For DTH experiments both DBA/1 and Balb/C mice could be used in order to analyse the impact of these iDCs-expanded Tregs on immune response. Preventive treatment with CD4<sup>+</sup>CD49b<sup>+</sup> T cells isolated from iDCs-vaccinated mice is performed just before sensitization by abdominal application of oxazolone. Five to 7 days after, challenge is performed by ear painting with the hapten and ear swelling could be monitored.

**Results:** We showed that the repetitive injections of iDCs just after the boost (days 21, 23, 25) did not protect the mice from arthritis whereas later injections at days 28, 30 and 32 attenuated progression of the disease and reduced bCII-specific antibody response. Moreover, a single injection of 100 000 Tregs at day 28 was able to delay disease onset and decrease the severity of the disease. We clearly demonstrated in this study, the impact of these induced-Tregs on the immune response since a dose dependent anti-inflammatory effect was observed on oxazolone model of DTH.

**Conclusion:** All these results suggest that the use of iDC vaccination in an inflammatory setting has to be carefully addressed. We demonstrated in this study the high therapeutic potential of the iDCs-induced Tregs on established arthritis in the CIA model. Moreover, the results obtained in the DTH model suggest a regulatory potential in others autoimmune diseases.

## 053 RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS INDUCE THE DIFFERENTIATION OF MONOCYTES INTO MACROPHAGES AND INTO CD1a<sup>+</sup> DC TO A LESSER EXTENT

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Monocytes represent a large pool of circulating precursors that can differentiate into macrophages or dendritic cells (DC). In rheumatoid arthritis (RA) synovium there is a dense infiltrate of CD1a<sup>+</sup> DC and macrophages, where they are found in close association with synovial fibroblasts. Therefore, we postulated that fibroblasts may represent a critical element in the control of APC differentiation.

Peripheral blood monocytes were cultured in the presence or in the absence of supernatants derived from RA synovial fibroblasts (RASf; 1 week culture supernatants) at different concentrations (12.5%, 25% and 50%)  $\pm$  GM-CSF  $\pm$  IL-4, for 3 or 6 days. In parallel, monocytes were seeded on RASf-containing plates (co-cultures) for 3 or 6 days. At the indicated time-points, medium and cells were harvested and FACS analysis was performed (CD1a, CD1c/BDCA1, BDCA2/CD303, CD14, CD163, HLA-DR and ChemR23).

Supernatants from cultured RASf induced the differentiation of monocytes into macrophages (CD163<sup>+</sup>), in a dose- and time-dependent manner and resulted in high expression of ChemR23. Moreover, RASf supernatants induced, to a lesser extent, the expression of CD1c and BDCA2/CD303. Interestingly, the induction CD1a expression (DC) was also patient dependent (expressions that varied from 10 to 60%). Increased expression of CD163 and ChemR23 (compared to monocyte cultures with RASf supernatants) was seen when monocytes were co-cultured with RASf, while DC generation (CD1a expression) was inhibited. The molecules responsible for the above mentioned differentiation are currently under investigation.

RA synovial fibroblasts have the capacity to skew the differentiation of monocytes into macrophages and DC to a lesser extent. Macrophage differentiation might be dependent not only on fibroblast-derived soluble factors but also macrophage-fibroblast cell-cell contact. Thus, RA fibroblasts may regulate local macrophage differentiation, cells that are known to contribute to disease pathogenesis.

## 054 BETA 2 GLYCOPROTEIN I ( $\beta$ 2GPI) BINDS PLATELET FACTOR 4 (PF4): IMPLICATIONS FOR THE PATHOGENESIS OF ANTIPHOSPHOLIPID SYNDROME

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**Background:** Antiphospholipid syndrome (APS) is an autoimmune acquired thrombophilia characterized by arterial or venous thrombosis and fetal loss. It has been suggested that anti- $\beta$ 2GPI antibodies activate endothelial cells and platelets by stimulating them to express adhesion molecules and tissue factor and to secrete proinflammatory cytokines.

Platelet factor 4 (PF4 or CXCL4) is a platelet a-granule protein that is a member of the CXC chemokine family. Initial investigations focused on possible procoagulant roles for PF4 in platelet function and plasmatic coagulation. Furthermore PF4 is implicated in the pathophysiology of heparin-induced thrombocytopenia (HIT) syndrome which shares clinical manifestations with APS.

**Aim:** To reveal membrane proteins from platelets, that can act as potential adaptor molecules of  $\beta 2$ GPI.

**Materials and methods:**

**Platelet membrane proteins purification:** Isolated platelets from 3 healthy donors and 1 APS patient were initially treated with neuraminidase. According to a specialized protocol (Moebius, Mol Cell Prot.2005) for purification of platelet crude membranes, only membrane proteins were extracted.

**$\beta 2$ GPI purification:**  $\beta 2$ GPI was isolated from sera of APS patients, using a combination of cardiolipin-affinity chromatography and HPLC.

**Affinity chromatography using CNBr-Sepharose- $\beta 2$ GPI:**  $\beta 2$ GPI was coupled in CNBr-sepharose and platelet membrane proteins were passed through the column. The eluted proteins were electrophoresed, stained with Coomassie or silver and analyzed with mass spectrometry.

**Saturation binding of biotinylated PF4 to  $\beta 2$ GPI coated onto microtiter plates:** The wells were coated with  $\beta 2$ GPI or  $\beta$ -lactoglobulin (10  $\mu$ g/ml) and incubated overnight. The plates were blocked with 4% BSA-PBS and 50  $\mu$ l of various concentrations (0–5  $\mu$ g/ml) of biotinylated PF4 was added and incubated overnight. Streptavidine-peroxidase was added and after OPD substrate solution, the optical density was measured.

**Competitive inhibition assay:** Pre-incubated biotinylated PF4 with increasing concentrations of  $\beta 2$ GPI, was added onto  $\beta 2$ GPI coated plates, as described above.

**Results:** The electrophoresis of the eluted materials from the  $\beta 2$ GPI-Sepharose column revealed 3 protein bands of 45 kDa, 18 kDa and 9 kDa which represent actin, myosin light chain and PF4 respectively, according to the mass spectrometry analysis. Taking into account that actin and myosin are extremely sticky molecules which usually form non-specific interactions, we studied the binding of PF4 to  $\beta 2$ GPI.

The results from the saturation binding experiments demonstrate that PF4 bound to  $\beta 2$ GPI with high affinity. The level of the inhibition reaches to 80% and confirms the specificity of the interaction.

**Conclusion:**  $\beta 2$ GPI selectively binds PF4 from purified platelet membrane proteins and this interaction is confirmed by in vitro binding assays. This result indicates a possible interaction between  $\beta 2$ GPI and PF4 in platelets, which may explain platelet activation of APS.

**055 WITHDRAWN**

**056 SYNERGISTIC COSTIMULATION OF CD4+CD28- T CELLS IN PATIENTS WITH RHEUMATOID ARTHRITIS BY DNAM-1, NKG2D AND 2B4**

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Effector T cell responses can be modulated by competing positive or negative signals transduced by NK cell receptors. In the CD4+ T cell population, the expression of NK receptors is primarily found in the CD4+CD28- T cell subset. These T cells are frequently found in rheumatoid arthritis and other inflammatory disorders, suggesting that ligation of NK receptors may promote autoimmune reactions. Here we aimed to dissect the phenotype and function of NK receptor-expressing CD4+CD28- T cells in patients with rheumatoid arthritis. By analyzing a broad array of NK receptors on CD4+CD28- T cells we found a significant expression of 2B4, DNAM-1 and NKG2D. While none of the receptors alone costimulated the response of primary CD4+CD28- T cells to suboptimal levels of anti-CD3 stimulation, pair-wise combinations of 2B4 with DNAM-1 and/or NKG2D lead to a synergistic increase of effector functions. Moreover, using multi-parameter flow cytometry, we demonstrate that such co-ligation lead to an increased magnitude in overall responsiveness rather than changes in qualitative aspects of the functional response. Altogether these results demonstrate a complex pattern of synergy in the NK

receptor-mediated modulation of functional responses by primary CD4+CD28- T cells in rheumatoid arthritis. This may have consequences for the inflammatory responses imposed by these cells, thus influencing disease manifestations.

**057 HIGH MOBILITY GROUP BOX CHROMOSOMAL PROTEIN-1 (HMGB-1) IS EXPRESSED IN IDIOPATHIC INFLAMMATORY MYOPATHIES AND MEDIATES MUSCLE WEAKNESS**

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**Background:** Idiopathic inflammatory myopathies (IIMs) are characterised by muscle weakness, inflammatory cell infiltrates, and major histocompatibility complex (MHC) class I expression in muscle fibres. We have earlier showed that high mobility group box chromosomal protein-1 (HMGB-1) which is a potent pro-inflammatory cytokine is expressed in muscle tissue with inflammatory cell infiltrates of IIM patients. Here we aimed to test the hypothesis that HMGB-1 could induce MHC class I expression in muscle fibres and thereby be an early molecule in the inflammatory cascade in myositis. We also wanted to investigate whether HMGB-1 by itself could cause muscle weakness.

**Methods:** We investigated HMGB-1 expression in IIM patients in different phases of disease with or without inflammation in muscle tissue. The results were correlated with the expression of MHC class I in muscle fibres and with regenerating fibres. In addition, adult muscle fibres from healthy wild-type mice were incubated with different concentrations of recombinant HMGB-1 to investigate if HMGB-1 could induce MHC class I expression as well as muscle weakness.  $\text{Ca}^{2+}$  release was used as a surrogate marker for muscle force. Moreover, the activity of a known receptor for HMGB-1, the receptor for advanced glycosylated end products (RAGE), was also investigated by using RAGE-/- mice and wild-type controls.

**Results:** In patients with short disease duration and without inflammatory infiltrates, HMGB-1 was expressed in the myoplasm of a large number of muscle fibres, greatly outnumbering the fibres with MHC class I expression and regenerating fibres. HMGB-1 was also expressed in patients with inflammatory infiltrates and in patients in a chronic late phase of disease without inflammatory cells; however, there was no difference between the number of fibres expression HMGB-1 and MHC class I. Recombinant HMGB-1 induced reversible up-regulation of MHC class I in muscle fibres from wild-type mice and irreversibly impaired  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum during induction of fatigue in a concentration dependent manner and was not reversible when HMGB-1 was washed out. The HMGB-1 induced muscle weakness was independent of RAGE expression.

**Conclusion:** Our data suggest that the ubiquitous molecule HMGB-1 may induce MHC class I in muscle fibres and is directly involved in the pathogenesis of muscle weakness by affecting  $\text{Ca}^{2+}$  release. Moreover, HMGB-1 not only initiates irreversible muscle weakness but is also widely expressed in patients with IIM in an early phase of disease without inflammation, making HMGB-1 a potentially interesting target for therapy in these disorders.

**058 THE ROLE OF ROR- $\gamma$ T IN REGULATING IL-17 GENE EXPRESSION**

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**Background:** Interleukin (IL)-17 is a potent pro-inflammatory cytokine that plays a critical role in autoimmune inflammatory disease models in mice and is likely involved in the pathogenesis of human rheumatoid arthritis (RA). In mice, IL-17 producing CD4+ T



cells are identified as a distinct subset of T helper cells, Th17, that are induced to differentiate by IL-6 and transforming growth factor (TGF)- $\beta$ . IL-23 maintains Th17 clonal expansion. Recently, retinoic acid receptor related orphan nuclear receptor (ROR)- $\gamma$ t was implicated as a master transcription factor for IL-17 gene expression in that Th17 cells could not be generated from ROR $\gamma$ t knockout CD4<sup>+</sup> T cells.

**Objectives:** To determine whether over-expression of ROR $\gamma$ t augments Th17 generation and to assess how ROR $\gamma$ t induces IL-17 gene expression in CD4<sup>+</sup> T cells.

**Methods:** CD4<sup>+</sup> T cells were isolated from the lymph nodes and spleens of wild-type (WT) or ROR $\gamma$ t transgenic (Tg) mice and were differentiated toward either Th1 or Th17. Production of IL-17 was quantified by intracellular staining and by ELISA. Predicted ROR $\gamma$ t binding sites in the il17 locus were determined by a bioinformatics approach and primers flanking these sites synthesized. ROR $\gamma$ t binding to the putative sites of the IL-17 gene promoter was analyzed by using chromatin immunoprecipitation (ChIP) assay with quantitative PCR.

**Results:** ROR $\gamma$ t Tg mice showed no baseline clinical abnormality. Forced expression of ROR $\gamma$ t in CD4<sup>+</sup> T cells in Tg mice did not induce spontaneous production of IL-17. When stimulated in vitro with anti-CD3/CD28 but no exogenous cytokines, ROR $\gamma$ t Tg CD4<sup>+</sup> T cells produced 10-fold more IL-17 than non-ROR $\gamma$ t Tg CD4<sup>+</sup> T cells, but produced 30–40% less interferon- $\gamma$ . TCR stimulation of ROR $\gamma$ t Tg CD4<sup>+</sup> T cells was absolutely required for IL-17 production and could not be bypassed by the addition of IL-6/TGF- $\beta$  or IL-23. Three potential ROR binding sites containing the consensus core motif AGGTCA were found within 3 kb of the IL-17 promoter region. ChIP with anti-ROR $\gamma$ t revealed that ROR $\gamma$ t bound to the proximal 200 bp of the IL-17 promoter in Th17, but not in Th1 polarized CD4<sup>+</sup> T cells.

**Conclusions:** Over-expression of ROR $\gamma$ t in CD4<sup>+</sup> T cells does not initiate spontaneous IL-17 gene expression but markedly enhances IL-17 production in response to TCR stimulation in the absence of exogenous cytokines. When ROR $\gamma$ t is over-expressed, TCR ligation is sufficient to induce IL-17 expression and IL-6/TGF- $\beta$  cannot bypass TCR signalling. Studies are ongoing to determine whether over-expression of ROR $\gamma$ t in CD4<sup>+</sup> T cells of Tg mice will exacerbate experimental arthritis. ROR $\gamma$ t appears to induce IL-17 production by direct regulation of the proximal IL-17 promoter in Th17, but not Th1 polarized CD4<sup>+</sup> T cells. Understanding of Th17 transcriptional regulation may provide new approaches to attenuate IL-17 production in RA and related disorders.

## 059 USING T-CELL SUBSET AND Treg TO DEFINE IMMUNOLOGICAL REMISSION POST TNF-BLOCKADE

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Biologic therapies show clear benefit for a significant proportion of patients and clinical remission defined by a DAS28<2.6 can be achieved in patients with early and established RA. In patients with early RA, TNF antagonism can also induce a sustainable clinical remission long after cessation of therapy. However, in patients with established RA, despite clinical remission, therapy is often continued indefinitely. We have identified a particular CD4<sup>+</sup> T-cell subset in RA, called “inflammation related cells” (IRC) with potential pathologic activity.<sup>1,2</sup> In DMARDs induced remission in established RA, these cells are allowed to persist by becoming “dormant”. Their persistence, however, predicts relapse.<sup>3</sup> We also showed reduced regulatory T-cell (Treg) frequency in early RA.<sup>4</sup> Following TNF-blockade, Treg increase and their functions are improved.<sup>5</sup> In contrast, in DMARDs induced remission, we did not observe a consistent increase in Treg frequency.<sup>4</sup> The aim of this study is to determine whether Treg and IRC frequencies could be used to objectively assess clinical remission and establish whether therapy can be safely withdrawn.

**Methods:** Patients achieving clinical remission (DAS28<2.6) for at least 6 months were recruited following 12 month anti-TNF therapy versus placebo treatment in early RA (n=24) and in established RA on anti-TNF (n=35). 10 patients in the established RA cohort stopped therapy after 6 months. We used advanced 6 colours flow cytometry to measure the frequency of IRC and Treg in blood samples. Cytokine analysis was also used.

**Results:** The frequency of Treg defined as FoxP3<sup>+</sup> cells is similar in established disease achieving remission whether patients stopped or not therapy. As compared to established RA, frequency is higher in early RA post TNF-blockade (p=0.05) and compared to placebo (p=0.01). Furthermore Treg frequency is higher in established disease with no subsequent flare (p=0.05) compared to patient destined to flare. The frequency of IRC is similar in established disease, stopped or not. Frequency of IRC is reduced to a higher extent in early compared to established disease or in the placebo group. Higher frequency of IRC can also predict flare (p=0.008). Preliminary data indicate that IL-10 levels are higher in TNF-blockade induced remission in established disease with no subsequent flare (p<0.10) whereas Rantes is lower in the same group (p=0.05).

**Conclusion:** These preliminary data indicate that immunological remission could be defined using low frequency of IRC, high frequency of Treg and higher circulating IL-10. We are currently correlating these results with imaging data to establish a complete remission picture. Achieving this status could help deciding whether or not therapy could be withdrawn safely.

1. Ponchel, et al. 2002 *Blood*.
2. Ponchel, et al 2005 *ART*.
3. Burgoyne, et al. 2007, *ARD*.
4. Lawson, et al. 2006, *Rheumatology*.
5. Ehrenstein, et al. 2004, *JEM*.

## 060 INTRINSIC NEGATIVE REGULATION OF PROINFLAMMATORY T HELPER TYPE 1 RESPONSES

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T helper type 1 (Th1) have been shown to be effective initiators of chronic inflammation in tissues expressing their cognate (auto)-antigen. Upon initial activation in the presence of the cytokine interleukin-12 (IL-12), Th1 cells are stably imprinted for the expression of interferon- $\gamma$  (IFN $\gamma$ ) by epigenetic modification of the ifn $\gamma$  gene and expression of the transcription factor T-bet. However, Th1 cells also exhibit regulatory potential. IFN $\gamma$  itself may limit inflammation due to the induction of expression of inducible nitric oxide synthase, and due to inhibition of the development of Th17 cells. Recently it was shown that the expression of IL-10 by Th1 lymphocytes represents one of the major physiological regulatory mechanisms of immune reactions. Here, we have analysed the molecular requirements for IL-10 expression in effector and memory Th1 lymphocytes. Induction of IL-10 expression in Th1 lymphocytes depends on initial costimulation with IL-12, and its downstream transcription factor Stat4. Isolation of Th effector and memory cells ex vivo according to secretion of IL-10, by the cytometric cytokine secretion assay revealed that upon culture in vitro, these cells do not memorize expression of IL-10 upon further restimulations. In these cells, the IL-10 gene is not detectably imprinted epigenetically, and IL-10 re-expression remains conditional on continued costimulation with inducing signals, such as IL-12. This finding suggests a Th1 intrinsic regulatory feedback mechanism and an anti-inflammatory role for IL-12 in secondary immune responses. In memory Th1 cells IL-10 expression remains dependent on IL-12, while reexpression of IFN $\gamma$ , itself a strong inducer of IL-12, is independent of the original inducer IL-12. Therapeutic targeting of regulatory IL-10 expression

in chronic rheumatic inflammation will have to consider these conditional requirements.

Using transcriptional profiling of once and repeatedly activated murine Th1 and Th2 cells, we have identified the basic helix-loop-helix transcription factor Twist1 whose expression is specifically induced in Th1 cells by IL-12. Expression of twist1 in Th1 cells increases with the number of activation cycles. Accordingly, in peripheral human Th cells expression of twist1 increases along the differentiation pathway, namely from CD45RA+ naïve Th cells to terminally differentiated CD45RA- CCR7- CD27- effector memory Th cells. Th cells isolated from the site of inflammation of patients suffering from rheumatic inflammation, Crohn's disease and ulcerative colitis express high levels of twist1, but not Th cells isolated from healthy tissue. Twist1 downregulates the production of the pro-inflammatory cytokines IFN $\gamma$ , IL-2 and TNF $\alpha$ , while not affecting IL-10 expression. Twist1 expression may represent an attempt at intrinsic downregulation of pro-inflammatory effector function, but which, in contrast, could contribute to maintenance and chronification of Th1 mediated inflammation.

### 061 ACTIVATION OF BONE MORPHOGENETIC PROTEIN SIGNALLING IN RHEUMATOID ARTHRITIS

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**Introduction:** Increasing evidence supports a role for bone morphogenetic proteins (BMPs) in joint homeostasis, destruction, repair and remodelling. Expression of different ligands has been demonstrated in synovium from healthy controls and from patients with rheumatoid arthritis (RA). Little is known about the function of these growth factors and cytokines and their respective target cells in the healthy and arthritic joint. Here, we study activation of BMP signalling in rheumatoid arthritis patients and characterize the target cells in the synovium.

**Methods:** Synovial biopsies from patients with RA were obtained by needle arthroscopy. Healthy synovium was obtained post-mortem from multi-organ donors. Expression of intracellular signalling molecules involved in the BMP pathway (receptor regulated SMAD-1/5/9 and inhibitory SMAD-6/7) were quantified using real-time quantitative PCR. Phosphorylation of the receptor regulated SMADs was studied by Western blot and immunostaining. Positive cells were further identified by double staining for CD3, CD20, CD68, CD138, CD90, alpha smooth muscle actin (SMA), endoglin (CD105) and von Willebrand factor (vWF). In addition, tissues from early RA patients taken before and 26 weeks after treatment with infliximab and methotrexate (n = 5) were evaluated for changes in the absolute and relative number of BMP responsive cells.

**Results:** No significant differences were observed in receptor-regulated and inhibitory SMAD mRNA levels between normal and RA synovia. Western blot for P-SMAD-1/5/9 showed an overall increase of active BMP signalling in synovium of RA patients compared to normal synovium protein extracts when corrected for synovial tissue weight. However, the relative amount of P-SMAD-1/5/9 compared to total SMAD-1/5/9 was not different between patients with RA and controls. Different P-SMAD-1/5/9 positive cell populations were identified in distinct compartments of the RA synovium, in particular in the perivascular, sublining and lining cells. P-SMAD-1/5/9 positive perivascular cells were alpha-SMA positive and located around vWF and endoglin positive endothelial cells. Some CD90 positive synovial fibroblasts were P-SMAD-1/5/9 positive, as was part of the CD68 positive synovial cells but other cells of the haematopoietic lineage showed no SMAD-1/5/9 phosphorylation. Treatment with infliximab and methotrexate improved synovial inflammation, reduced synovial cellularity and total number of P-SMAD-1/5/9 positive cells, but did not affect the percentage of BMP responsive cells.

**Conclusions:** BMP signalling is activated in synovium of RA patients but also in controls. BMP-activated cells belong to distinct compartments. The perivascular niche includes mesenchymal cells and could be of interest to further define progenitor cells in the synovium. Treatment with anti-TNF did not abolish active BMP signalling, suggesting that this pathway is more likely to be involved in tissue homeostasis than in inflammation.

### 062 IgG-AUTOANTIBODIES FROM SCLERODERMA PATIENTS INDUCES INTERLEUKIN-1 $\alpha$ IN HUMAN KERATINOCYTES

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**Introduction:** Tissue fibrosis caused by the excessive deposition of extracellular matrix is a common feature of many connective tissue diseases, notably scleroderma (systemic sclerosis; SSc). Evidence suggests that complex intercellular interactions involving immune cells, endothelial cells and fibroblasts are important pathogenic events. However, the role of cellular events involving epithelial cells in initiating and maintaining fibrosis has not been extensively explored. The epidermal keratinocyte is a major source of pro-inflammatory and pro-fibrotic mediators. Antibodies directed against these cells have been reported in a number of other autoimmune diseases, and appear to lead to keratinocyte activation and secretion of potent soluble inflammatory/fibrotic mediators. We have previously shown that in early SSc the epidermis exhibits a phenotype resembling that observed in wound healing, and expresses a number of markers characteristic of epidermal differentiation.

**Objective:** We studied the presence of anti-keratinocyte antibodies in SSc and examined the influence of the keratinocyte autoantibodies on the secretion of IL-1 $\alpha$ .

**Method:** Sera were obtained from 10 diffuse SSc and 10 healthy controls and evaluated for antibody binding to keratinocyte cells by cell-based ELISA. IgG was purified from 3 SSc patient and 3 controls selected from cell based ELISA assay. Pre-incubation of IgG from SSc and from control with keratinocytes was assessed for intracellular and extracellular expression of IL-1 $\alpha$ . Interaction of IgG with keratinocyte binding and internalization was assessed using immunofluorescence. Biopsy sections were stained for human IgG. IL-1 $\alpha$  expression in scleroderma epidermis was assessed by ELISA.

**Results:** We found that IgG purified from SSc patients bound to nucleolar antigens in keratinocytes compare to that of control IgG. Furthermore, pre-treatment of human keratinocyte cells in vitro with purified IgG from SSc sera led to the secretion of IL-1 $\alpha$  from keratinocytes. Staining of anti-human IgG in biopsy section from scleroderma patients showed cell membrane staining in the epidermis compared to control biopsies. In addition, IL-1 $\alpha$  is over-expressed SSc epidermis compare to control.

**Conclusion:** We have demonstrated the over-expression of IL-1 $\alpha$  in SSc epidermis compared to that of control epidermis. Treatment of keratinocytes with IgG purified from the sera of patient with SSc resulted in a time dependent increase in the secretion of IL-1 $\alpha$ . These data suggest over-expression of IL-1 $\alpha$  by epidermal cells due to antibody-mediated activation plays a key role in the abnormal function of both dermal and epidermal cells in SSc.

### 063 CHARACTERIZATION OF A NOVEL REGULATORY T CELL SUBSET IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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**Introduction:** CD4+CD25+Foxp3+ regulatory T cells (Treg) specialize in the suppression of immune responses and are important for sustaining peripheral tolerance towards self-structures. In autoimmune conditions, however, Treg or activated T cells with specific phenotypic characteristics might arise. In this study

we performed phenotypic and functional analyses of a distinct CD4+Foxp3+ T cell population that lacked CD25 expression (CD4+CD25-Foxp3+) in patients with autoimmune diseases as compared to healthy controls (HC).

**Methods:** The phenotype of peripheral blood T cells was analyzed by flow cytometry in patients with systemic lupus erythematosus (SLE), systemic sclerosis (SSc), rheumatoid arthritis (RA) and HC. The percentage of CD4+CD25-Foxp3+ T cells was correlated with clinical data, the daily cortisone dose and the SLE disease activity index (SLEDAI). In addition CD4+CD127-CD25- T cells, as a surrogate population for CD4+CD25-Foxp3+ T cells defined by surface marker molecule expression, were isolated from SLE patients by fluorescence activated cell sorting (FACS) and analyzed for their suppressive capacity.

**Results:** Increased proportions of CD4+CD25-Foxp3+ T cells were observed in patients with SLE as compared to patients with SSc, RA or HC. CD4+CD25-Foxp3+ T cells phenotypically resembled Treg rather than activated T cells as determined by the expression of marker molecules such as CD27, CD45RO, CD62L, CD95, CTLA-4 or GITR and CD69, CD71 or HLA-DR, respectively. A significant correlation was observed for proportions of CD4+CD25-Foxp3+ T cells with the SLEDAI score and the daily cortisone dose. Finally, isolated CD4+CD127-CD25+ T cells and CD4+CD127-CD25- T cells, which both expressed Foxp3, effectively suppressed T cell proliferation in vitro.

**Conclusion:** Increased proportions of CD4+CD25-Foxp3+ T cells were observed in SLE patients as compared to HC. These cells phenotypically and functionally resemble Treg and might represent an attempt of the immune system to counter-regulated self-reactivity in active SLE patients.

#### 064 LYMPHOID NEOGENESIS AND LYMPHATIC ANGIOGENESIS IN RHEUMATOID SYNOVITIS: CLINICAL AND RADIOGRAPHIC CORRELATES

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**Background:** Infiltrating mononuclear cells can acquire different degrees of organisation in the inflamed synovium in rheumatoid arthritis (RA), with the possible acquisition of lymphoid neogenetic features. While synovial lymphoid neogenesis is considered to play a relevant bio-pathological role in RA, its clinical significance is still debated. Here we investigated the clinico-pathological correlates of synovial lymphoid neogenesis in a large cohort of RA patients.

**Materials and methods:** Synovial samples were collected from 105 RA patients. The main demographic, clinical, laboratory and radiographic parameters were recorded. The inflammatory infiltrate was classified in diffuse/aggregational by two independent observers on H&E sections and quantified (0–3). An independent aggregational and diffuse score was also assigned to each specimen. T and B lymphocytes and plasma-cells were investigated by immunohistochemistry (IHC) for specific markers and quantified. Lymphoid neogenetic features were also assessed. The presence of synovial lymphatic vessels was analysed using the specific marker LYVE-1.

**Results:** The classification of samples in diffuse/aggregational was highly discordant between observers because of the co-existence of areas of diffuse infiltration and mononuclear aggregates in 25/105 samples (23.8%). By assigning an independent diffuse and aggregational score to each specimen, the diffuse infiltrate showed a positive correlation with ESR and CRP values ( $p < 0.001$ ), while the aggregational score did not significantly correlate to any of the parameters analysed. Further IHC evaluation revealed a positive correlation between diffuse CD3+ T cells and ESR, CRP and DAS ( $p < 0.05$ ) and between CD20+ B aggregate score and radiographic erosions ( $p = 0.008$ ). CD138+ plasma cells did not show any

significant correlation. No association was observed between CD21+ FDC networks (13% of the samples) and serum auto-antibodies. The development of lymphatic vessels was observed in 28/105 cases (26.7%), and in particular in 9/24 (37.5%) diffuse, 0/56 (0%) aggregate and 19/25 (76%) mixed (i.e. co-existence of diffuse and aggregational infiltration) samples. In these latter, lymphatic vessels were associated to diffuse areas or poorly organised cell clusters. Furthermore, the lymphatic network correlated with higher histological inflammatory scores and CRP values ( $p < 0.001$ ).

**Conclusions:** Our results demonstrate that, in RA, a diffuse synovial lymphoid infiltrate represents the histopathological correlate of active inflammation, while aggregates of B cells are observed in erosive disease. Furthermore, the organisation of infiltrating mononuclear cells within lymphoid follicles seems to be a dynamic process, possibly modulated by the development of a lymphatic network in response to inflammation. The analysis of the synovial lymphoid pattern and of factors involved in its modulation may thus provide important information in the clinical assessment of RA patients.

#### 065 HETEROGENEITY OF RHEUMATOID ARTHRITIS BASED ON MOLECULAR, IMMUNOHISTOCHEMICAL AND CLINICAL MARKERS

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**Introduction:** Previously, we identified different pathological subtypes of rheumatoid arthritis (RA) by gene expression profiling of RA synovial tissues from patients undergoing total joint replacement surgery. In the current study we aimed to confirm these results in synovial tissue biopsies obtained by arthroscopy and to compare the molecular subclassification with classification based on histology.

**Methods:** cDNA microarrays were used to analyze the gene expression profiles of synovial tissue samples obtained by arthroscopic surgery ( $n = 17$ ). Synovial tissues were stained for the cellular markers CD163, CD22, CD3, CD38 and CD68. In addition, tissues were stained for E-Selectin, TNFalpha, VCAM and ICAM. For both the molecular and immunohistochemical subclassification, an unsupervised two-way hierarchical cluster analysis was used.

**Results:** Based on the tissue gene expression profiles this molecular approach revealed the presence of at least two subgroups of patients i.e. a high and a low inflammatory subgroup. These data confirm our previous findings on synovial tissues obtained during total joint replacement surgery. We compared the molecular subclassification with the unsupervised two-way cluster analysis based on immunohistochemical data. Strikingly, the subclassification into two groups based on the data of the two different approaches was an exact match.

Clinically, the patients with a high inflammatory tissue type displayed a higher disease activity score, more tender joints, higher VAS, higher ESR, higher CRP levels and more thrombocytes compared to the low inflammatory group.

**Conclusion:** The molecular subclassification of RA using arthroscopic biopsies is in line with our previous data. In addition, these data show that tissue subtyping based on gene expression profiles reflects inflammatory processes detected at the protein level by immunohistochemistry and can therefore be of inestimable value for finding pathogenic mechanisms involved in the disease process.

#### 066 CTLA-4 DIRECTLY INHIBITS OSTEOCLAST FORMATION

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CTLA-4 is a regulator of co-stimulation and inhibits the activation of T cells through interfering with the interaction of CD80/86 on



antigen presenting cells with CD28 on T cells. CTLA-4 binds to the surface of antigen presenting cells, such as dendritic cells and monocytes through CD80/86. Monocytes can differentiate in osteoclasts, the primary bone resorbing cells. Herein, we investigated whether binding of CTLA-4 affects the differentiation of monocytes into osteoclasts in vitro and in vivo. We show that CTLA-4 dose dependently inhibits RANKL- as well as TNF-mediated osteoclastogenesis in vitro without presence of T cells. Furthermore, CTLA-4 was effective to inhibit TNF-induced osteoclast formation in a non-T cell dependent TNF-induced model of arthritis as well as the formation of inflammatory bone erosion in vivo. These data suggest that CTLA-4 is an anti-osteoclastogenic molecule, which directly binds osteoclast precursor cells and inhibits their differentiation. These findings are an attractive explanation for the anti-erosive effect of abatacept, a CTLA-4-Ig fusion protein used for the treatment of RA.

#### 067 FLOW CYTOMETRY ANALYSIS OF THE SYNOVIUM: A QUANTITATIVE METHOD OF ANALYSIS

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**Background:** Analysis of synovial tissue allows studying mechanisms of chronic arthritis. Immunohistochemistry is most commonly used to investigate the cell populations involved in the disease and responses to therapy. However, this technique is laborious, time-consuming, and not easy to standardize between centres. We evaluated multi-colour flow cytometry as a tool to identify different cell populations present in the synovium of patients with chronic arthritis and orthopedic problems.

**Methods:** Synovial biopsies from 10 patients with mechanical knee problems ("ORT samples") and 20 patients with chronic arthritis ("REU samples") were collected. 7 REU patients had rheumatoid arthritis (RA) and 13 patients had spondyloarthritis (SpA). All tissues were treated with a standardized digestion procedure, composed of a mechanical disruption step by mincing the tissue and an enzymatic step comprising treatment with hyaluronidase (15 minutes) and collagenase IV (2 hours) under continuous rotation. The freshly isolated cells were stored in liquid nitrogen before flow cytometry analysis. Viability staining with 7-AAD and binding of CD45 were used for primary analysis. Within the CD45-negative population, the CD90- (fibroblasts and endothelial cells) and CD34-positive (endothelial cells) populations were quantified. Within the CD45-positive population, the CD3- (T cells), CD20- (B cells) and CD14-positive (macrophages) populations were quantified. The impact of the isolation method on marker expression was investigated by subjecting PBMCs or cultured fibroblasts to the isolation procedure.

**Results:** The ORT donors had a mean age of 43 years and the REU donors had a mean age of 40.5 years. The mean cell count after isolation in ORT samples was 2,345 cells/mg with a viability of 53%. In REU samples, the yield was 20,444 cells/mg with 68% viability. The ratio of CD45-negative/CD45-positive cells was inverted in the REU-group compared to the ORT-group. Comparing the relative expression of CD3, CD14 and CD20, there was no difference between patients with SpA and RA. However, there was a higher proportion of CD34-positive cells in the SpA group compared to the RA group (respectively 40.5% and 18% of CD45-negative cells). The expression of CD20 and CD14, but not of CD3, CD34 and CD90, was somewhat affected by the isolation procedure. The mechanical component decreased the expression of both B cell markers CD20 and CD19 on PBMCs while the enzymatic component decreased the expression of CD14 and CD163.

**Conclusion:** Cells of the synovium can be quantified by multi colour flow cytometry. B-cell count is affected by rotation and macrophage count is influenced by the enzymatic component. The difference in CD34 positive cells between RA and SpA samples

confirms earlier observations made by immunohistochemistry and identifies flow cytometry as a useful method for tissue analysis.

#### 068 SCLERODERMA ALVEOLITIS: A VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) DEFICIENCY-RELATED LUNG DISEASE?

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**Background:** Vascular endothelial growth factor (VEGF) is abundantly expressed in adult lung tissue, and it is secreted by almost all the cell types found in airspace or lining. Although excess of VEGF leads to pathological consequences, underexpression also seems to have deleterious effects on the pulmonary vasculature and alveolar structure. Moreover, animal studies and clinical data support a protective role for VEGF in lung injury and decreased VEGF expression was found to be associated with impaired recovery from lung injury. So it has been suggested that VEGF within the lung may play a dual role, not only in contributing to the regulation of the alveolar-capillary permeability but also by promoting lung repair. VEGF resulted in measurable bronchoalveolar lavage fluid (BALF) of all normal subjects and was found to be reduced in patients with pulmonary fibrosis. Poor and discordant data have been reported about systemic sclerosis (SSc) lung involvement and serum or BALF VEGF concentration.

**Methods:** Plasma and BALF VEGF levels were analysed through ELISA (Biosource) in 47 SSc patients with evidence of lung involvement on high resolution computed tomography (HRCT) undergoing bronchoalveolar lavage (BAL). Pulmonary function tests (PFTs) and HRCT were repeated in all the patients after 14.8±5.9 months of follow up.

**Results:** BALF VEGF levels were significantly lower in the patients with alveolitis than patients without (median 274.14 ng/mL vs 1682.14 ng/mL;  $p=0.010$ ). BALF VEGF was detectable only in 4 (4.2%) of the patients with alveolitis and in 11 (47.8%) of the patients without (OR = 4.58(95% CI 1.07–22.3)). The significant association between lower BALF VEGF levels and alveolitis was further confirmed by the inverse correlation between BALF VEGF levels and the alveolar score on HRCT ( $p=0.037$ ,  $r=-0.31$ ), BALF total cell count ( $p=0.004$ ,  $r=-0.41$ ), macrophage count ( $p=0.028$ ,  $r=-0.32$ ) and lymphocyte count ( $p=0.035$ ,  $r=-0.31$ ). In addition the patients with undetectable BALF VEGF had a higher BALF neutrophil count ( $17.7\pm11.2$  vs  $4.8\pm9.3$ ,  $p=0.022$ ). The possible protective role of constitutive lung VEGF expression was confirmed by the association between lower BALF VEGF level and worsening of lung involvement at follow up. The patients with undetectable BALF VEGF levels presented a significant worsening in the interstitial score at follow up (T0  $7.3\pm1.8$  T1  $8.5\pm3.0$ ,  $p=0.045$  vs T0  $7.1\pm1.6$  T1  $7.6\pm2.5$ ,  $p=ns$ ). There was no significant association either between BALF VEGF levels and PFTs worsening at follow up or with age, disease duration and smoke habit. No correlation was found between BALF and plasma VEGF levels.

**Conclusions:** VEGF was found to be undetectable in the BALF of the majority of the SSc patients with lung involvement on HRCT. The patients with alveolitis showed the lowest BALF VEGF levels. Moreover undetectable BALF VEGF was associated with worsening of the interstitial score at follow up.

#### 069 SERUM LEVELS OF TNF FAMILY MEMBERS APRIL (A PROLIFERATION INDUCING LIGAND) AND BLYS (B LYMPHOCYTE STIMULATOR) ARE INVERSELY CORRELATED IN SYSTEMIC LUPUS ERYTHEMATOSUS

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**Objective:** Several studies suggest an implication of the two TNF-family members APRIL (A Proliferation Inducing Ligand) and BlyS

(B Lymphocyte Stimulator) in systemic lupus erythematosus (SLE). BlyS serum levels appear to be elevated in SLE patients, whereas opposite observations were reported for APRIL serum levels. We therefore performed a clinical study to clarify this issue.

**Methods:** Forty-three patients with SLE, responding to ACR's criteria and with positive anti-dsDNA antibodies once in their medical history, were included. Clinical examination was performed and disease activity evaluated by SLEDAI. Biological parameters including titres of autoantibodies (antinuclear antibodies, anti-dsDNA, anti-ENA, anti-phospholipid antibodies and rheumatoid factor) were measured in parallel to the clinical examination at the same visit. Serum APRIL and BlyS levels were assessed by ELISA and compared to serum levels of 27 healthy control subjects. Student's test was used for comparison between serum levels, Spearman's rank order test and Mann-Whitney analysis for correlation of parameters.

**Results:** For SLE patients, median age was 38.6 years (19–63) and median SLEDAI was 6 (0–30). Median serum levels of APRIL and BlyS were high with respectively 24 ng/ml and 1.7 ng/ml for SLE patients, and 4 ng/ml and 0.9 ng/ml for controls ( $p < 0.05$ ). APRIL serum levels showed inverse correlation with BlyS serum levels ( $r = -0.339$ ;  $p = 0.03$ ), tended to be inversely correlated with anti-double-stranded (ds) DNA titers ( $r = -0.261$ ;  $p = 0.09$ ), and associated with kidney manifestations ( $p = 0.103$ ). There was neither correlation between BlyS and APRIL serum levels and the positivity of anti-ENA, antiphospholipid antibodies or rheumatoid factor, nor with SLEDAI. Interestingly, serum APRIL levels inversely correlated with renal involvement in SLE patients ( $p = 0.03$ ). In a follow-up study at their second visit at 4 months, 27 patients showed an inverse correlation with BlyS ( $r = -0.398$ ;  $p = 0.03$ ) and anti-dsDNA ( $r = -0.408$ ;  $p = 0.03$ ) titers, as well as an inverse correlation of APRIL serum levels with SLEDAI ( $r = -0.408$ ;  $p = 0.01$ ).

**Conclusion:** APRIL may regulate BlyS effects and may be a protective factor in SLE patients, decreasing anti-DNA antibodies titres and renal involvement. APRIL, instead of being a therapeutic target, could be a relevant treatment in SLE patients. These results need to be confirmed by further studies.

## 070 PRELIMINARY RESULTS ON IGG GLYCOSYLATION PROFILES IN RHEUMATOID ARTHRITIS PATIENTS DURING PREGNANCY AND POSTPARTUM

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**Introduction:** Rheumatoid arthritis (RA) improves during pregnancy. The mechanism underlying this phenomenon is unknown. RA has been associated with abnormal glycosylation of immunoglobulins, namely with a reduction in galactosylation of the immunoglobulin G (IgG) N-glycans. IgG without galactose is called agalactosylated IgG (IgG-G0). It is also unknown whether changes in the percentage of IgG-G0 during pregnancy are causatively associated with pregnancy-induced remission of RA, or are rather an epiphenomenon. Within the cohort of the PARA-study (Pregnancy-induced Amelioration of RA), a large prospective study which follows women with RA during pregnancy and postpartum, we aim to investigate the changes in IgG-G0 during pregnancy in RA patients and controls, and whether these are associated with disease activity scores (DAS28).

**Materials and methods:** Serum from  $n = 152$  RA patients was collected at six or seven time points: if possible pre-pregnancy, during pregnancy in the first, second and third trimester and postpartum after 6 weeks, three and six months. Protein A purification of IgG was followed by trypsin digestion and purification of (glyco-)peptides by reverse-phase solid phase

extraction. Glycopeptides were analyzed by MALDI-TOF-MS in the automatic mode. Glycopeptides derived from IgG1 and IgG2 were differentiated on the basis of their peptide mass, and signal intensities were used for quantification. IgG1-G0 was normalized to the sum of IgG1-G0, IgG1-G1, and IgG1-G2. An analogous normalization was performed for IgG2-G0. Differences between time points were investigated by Wilcoxon rank test.

**Results:** Data are shown for the first  $n = 58$  RA-patients who were visited from pre-pregnancy onwards. Already in the first trimester the percentage of IgG1-G0 decreased significantly compared to pre-pregnancy levels (median 35.9% to 29.4%). This decrease continued until the third trimester (24.6%,  $p < 0.0001$ ). At 6 weeks postpartum, this percentage was significantly increased to 28.7%. At 6 months postpartum the IgG1-G0 levels were further increased (31.3.1%,  $p < 0.0001$ ). Data for IgG2-G0 show a similar pattern.

At present IgG-G0 serum levels of additional patients and healthy controls are being analyzed in association with DAS28. These data will be available shortly and hopefully presented at the EWRR.

**Discussion:** IgG-G0 glycosylation profiles in RA-patients were found to vary significantly during pregnancy and in the postpartum period, showing decreased levels of IgG-G0 during pregnancy and the reverse in the postpartum period. Ongoing future studies focus on extending the number of RA-cases and adding data of healthy controls. Finally the glycosylation profiles will be analyzed for association with RA-disease activity during pregnancy and postpartum.

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## Innate immunity, infection and inflammation

### Oral presentation

#### 071 EFFECTS OF TNF BLOCKADE BY INFlixIMAB ON INFLAMMASOME REGULATORS IN RHEUMATOID ARTHRITIS PATIENTS

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**Background:** The proinflammatory cytokines, interleukin (IL)-1 and tumour necrosis factor (TNF), are key molecules in the pathogenesis and persistence of rheumatoid arthritis (RA), with associated joint damage. Blockade of TNF in these patients, using current biological response modifiers, has had profound therapeutic effects with marked benefit to the majority of patients. RA has traditionally been described as being due to adaptive immune responses, but there has been a gradual appreciation of the role of innate immunity, initiating and maintaining subsequent adaptive responses. The NALP3 inflammasome is a highly conserved and specific response system, composed of a proinflammatory protein complex involved in regulation of innate immunity and the production of IL-1. This study investigates the expression and activation of various components of the NALP3 complex in RA patients and the effects of TNF blockade by infliximab on this pathway.

**Methods:** The RA patients studied ( $n = 19$ ) had all failed at least two traditional disease modifying anti-rheumatic drugs, before being initiated onto infliximab. Protein and mRNA expression analysis was carried out on peripheral blood mononuclear cells (PBMC), pre- and 2 hours post-infusion, at weeks 0, 2, and 14 for all 19 patients. The Disease Activity Score (DAS28) classified patients as being good, moderate ( $n = 9$ ) and non-responders ( $n = 10$ ) to infliximab, with a decrease in DAS28 of more than 1.2 indicating a good/moderate response. ASC (caspase-1-activating adaptor for IL-1 generation) expression in cytosolic fractions derived from PBMC was analysed by western blotting (WB), probing for the ASC

protein. Quantitative PCR was used to detect ASC and NALP3 messenger RNA levels from PBMC of these patients.

**Results:** Infliximab had marked effects on the expression of ASC, with significant increases in both gene and protein expression levels in responding patients; there was a significant increase in ASC mRNA between pre-infusion at week 2 and pre-infusion at week 14 ( $p = 0.036$ ). Furthermore, there was also a significant decrease in the NALP3 transcript levels in the responders, both prior to treatment (pre-week 0) as well as pre- ( $p = 0.012$ ) and post-infusion ( $p = 0.028$ ) at week 14.

**Conclusions:** Previously we have reported differences in expression levels of the inflammasome regulator, ASC, between RA patients treated with either infliximab or etanercept, with infliximab inducing changes in the ASC transcript and protein expression. We have confirmed these findings and now report on the effects of infliximab on NALP3 mRNA levels. In addition to its role as an adaptor protein in the NALP3 complex, ASC or TMS1 (Target of Methylation-induced Silencing 1) also induces other effects in inflammatory cells, independent of its adaptor role in the NALP3 inflammasome. Infliximab induces an increase ASC protein and a decrease in the NALP3 transcript levels in RA patients responding to infliximab, and this is not observed in RA patients who do not respond to infliximab therapy.

## Oral presentation

### 072 SYNERGY BETWEEN IL-32 AND BACTERIAL FRAGMENTS RESULTS IN CHRONIC DESTRUCTIVE ARTHRITIS

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**Introduction:** IL-32 might play an important role in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA) by inducing cytokines and chemokines like TNF $\alpha$ , IL-1 $\beta$  and IL-8. Moreover, it was demonstrated that IL-32 is highly expressed in RA synovial tissue and strongly correlates with synovial inflammation, TNF $\alpha$  and IL-1 $\beta$  expression. Furthermore, IL-32 synergizes with NOD1 and NOD2 ligands for IL-1 $\beta$  and IL-6 production, indicating a potential synergistic role between IL-32 and bacterial fragments.

**Objectives:** To investigate the synergistic role of IL-32 and bacterial fragments in the development of chronic destructive arthritis in mice.

**Methods:** For expression and targeting of IL-32 into mice knee joints, an adenoviral vector encoding for IL-32 (adIL-32) was developed. Before testing adIL-32 in vivo, adIL-32 was validated in vitro for IL-32 mRNA expression by quantitative PCR and protein expression by immunolocalization in 3T3 and HeLa cells. Subsequently, C57BL/6-wt mice were intraarticular injected with adIL-32 or control virus at day 0, followed by intraarticular injection of Streptococcus cell wall (SCW) fragments containing predominantly TLR2/NOD2 ligands at day 1. At day 2, synovial tissues were isolated and mRNA levels of TNF $\alpha$ , IL-6, and IL-1 $\beta$  were measured by quantitative PCR. Additionally, macroscopic scores were determined before isolating knee joints for histology at day 4 and 7.

**Results:** In vitro data showed that IL-32 mRNA and protein was expressed in 3T3 and HeLa cells. Furthermore, elevated mRNA levels of proinflammatory cytokines like TNF $\alpha$ , IL-6, and IL-1 $\beta$  were found in mice injected with adIL-32 and SCW fragments. Moreover, mice injected with adIL-32 in combination with TLR2/NOD2 ligands displayed severe arthritis, when compared to mice injected with control virus and SCW fragments. Subsequently, histological analysis showed, apart from enhanced numbers of inflammatory cells, strong proteoglycan depletion, severe cartilage destruction, and massive chondrocyte death in mice exposed to IL-32 and TLR2/NOD2 ligands. In contrast, control virus and SCW injected mice revealed to display a mild non-destructive arthritis.

**Conclusion:** Here we show that IL-32 in combination with TLR2/NOD2 results in notably higher mRNA levels of proinflammatory cytokines like TNF $\alpha$ , IL-6, and IL-1 $\beta$ . In addition, injection of adIL-32 and SCW fragments leads to chronic arthritis accompanied with severe joint destruction. The observed synergistic effect between IL-32 and TLR/NOD ligands identifies an important amplifying pathway in the development of chronic destructive arthritis. It implies that IL-32 antagonists may provide an important novel therapeutic target in RA.

## Oral presentation

### 073 FUNCTIONAL TLR9 IS GAINED IN B-CELLS AT PRE-B/IMMATURE STAGES OF B-CELL MATURATION IN BONE MARROW

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**Objectives:** Our recent data provide strong evidence of the presence of functional TLR9 in bone marrow (BM) B-cells isolated from rheumatoid arthritis (RA) patients. TLR9 agonists (unmethylated CpG but not control GpC oligonucleotides) enhance CD54 and CD86 expression, trigger pro-inflammatory cytokines (TNF $\alpha$ , IL-6) and proliferation and further differentiation of bone marrow B-cells. In this study we tested at which stage of B-cell development/maturation in bone marrow, the functional TLR9 is expressed.

**Methods:** Peripheral blood and bone marrow samples, obtained from RA patients during joint replacement surgery, served for PBMC and bone marrow mononuclear cells (BMMC) isolation. The expression of TLR9 protein on several subsets of B-cells, including Pro-B (CD19+, CD20-) and Pre-B (CD19+, CD20+) from PBMC and BMMC, was assessed by flow cytometry. Functionality of TLR9 was evaluated in B lymphocyte subpopulations, after their culture in vitro for 72 h in the presence of agonistic (CpG) or control (GpC) oligonucleotides, using CD86 staining and flow cytometric analysis.

**Results:** Flow cytometric analysis reveals that bone marrow contains three, clearly distinct, subpopulations of CD19+ B-cells: (1) CD19+,CD20-, (2) CD19+,CD20low, and (3) CD19+,CD20+, representing different stages of B-cell maturation: Pro-B, Pre-B and immature B-cells. The expression of TLR9 in these B-cell precursors parallels the increase of CD20 expression on BM B cells:  $32.3\% \pm 7.7$  in CD20- cells vs  $67.4\% \pm 7.6$  in CD20low cells vs  $84.2\% \pm 4.4$  in CD20+ cells, indicating that TLR9 is acquired at Pre-B/immature stages of B cell maturation in BM. The levels of TLR9 in BM CD20+ B cells are comparable to TLR9 expression in peripheral blood CD20+ cells at both percentage of positive cells ( $84.2\% \pm 4.4$  in BM vs  $89.9\% \pm 2.8$  in peripheral blood, and mean fluorescence intensity ( $57.5 \pm 3.6$  in BM vs  $64.0 \pm 5.7$ ) in peripheral blood. Comparable TLR9 levels in BM- and peripheral blood-derived CD20+ B cell populations suggest that, like peripheral blood B cells, BM B cells could respond to direct TLR9 stimulation. Indeed, responsiveness of bone marrow B-cells to TLR9 stimulation measured by enhancement of CD86 expression correlates with increased CD20 expression. It was interesting, however, that despite similar levels of TLR9 expression, the bone marrow CD20+ B cells responded better than their peripheral blood counterparts to CpG stimulation in vitro.

**Conclusions:** Although some TLR9 are present on Pro-B cells, the full expression of TLR9 are gained on pre-B/immature B stages of B-cell maturation in bone marrow. Since bone marrow CD20+ B cells respond to TLR9 agonist stimulation more vigorously than peripheral blood CD20+ cells, they may represent the first line of responders to blood borne bacterial DNA trafficking to bone marrow. Our results support the notion that bone marrow represents an important site for B-cell activation and may contribute to the pathogenesis of RA.



## 074 SHIFT FROM TLR2 TOWARDS TLR4 DEPENDENCY DURING THE DEVELOPMENT OF CHRONIC STREPTOCOCCAL CELL WALL-INDUCED ARTHRITIS

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Toll-like receptors (TLRs) may initiate innate and instruct adaptive immune responses in rheumatoid arthritis (RA) through recognition of microbial as well as endogenous ligands repeatedly found in arthritic joints. We studied the involvement of TLR2 and TLR4 in the development of chronic arthritis using reactivated streptococcal cell wall (SCW)-induced model in which the TNF-dependent macrophage-driven acute phase is shifted towards an IL-1/IL-17 dependent T cell-driven process during the chronic phase.

Reactivated SCW arthritis was induced by four repeated intraarticular injection of SCW fragments—known to directly signal through TLR2—in wild type (WT), TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice. Joint swelling, histopathology, and T and B cell responses were studied, and mRNA and protein expression of cytokines, chemokines, and markers of joint destruction were evaluated using quantitative polymerase chain reaction, multiplex bead array and immunohistochemistry.

The TLR2 dependency of the joint swelling in the acute phase was replaced by TLR4 dependency in the chronic phase, indicating that TLR4 takes over the function of TLR2 during chronic inflammation. Although macroscopic inflammation score of knee joints immediately after SCW injection was reduced in TLR2<sup>-/-</sup> mice, only TLR4<sup>-/-</sup> animals showed a sustained suppression of inflammation during the chronic phase. Histological analysis revealed less influx of inflammatory cells into the joints of both TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice. Interestingly, TLR4<sup>-/-</sup>, but not TLR2<sup>-/-</sup>, mice had significantly lower concentrations of IL-1 $\beta$  and IL-6, both in serum and patella washouts. Furthermore, the adaptive B cell response was lower in TLR4<sup>-/-</sup> mice, as these mice had less anti-SCW specific IgG1 and IgG3 antibodies in serum. While the development of SCW-specific T cell response was comparable in all groups, T cells from TLR4<sup>-/-</sup> mice produced markedly less IL-6 and IL-17 upon antigen specific stimulation, when compared to WT and TLR2<sup>-/-</sup> cells. mRNA expression of IL-1 $\beta$ , TNF $\alpha$ , IL-23, iNOS, and matrix metalloproteinases (MMP) 3, 9 and 13 was strongly suppressed in synovial tissues of TLR4<sup>-/-</sup> mice in the chronic phase, while TLR2<sup>-/-</sup> synovial tissue showed only a slight reduction in IL-1 $\beta$  and TNF $\alpha$ . Immunohistochemical staining of the joint sections confirmed significantly lower expression of IL-1 $\beta$ , the osteoclast marker Cathepsin K and the MMP-specific aggrecan neopeptide VDIPEN in TLR4<sup>-/-</sup> joints.

These data demonstrate a shift from TLR2 towards TLR4 dependency of chronic SCW arthritis model. TLR2 dependency of the acute phase is due to its direct activation by SCW fragments. TLR4 activation becomes prominent during the chronic phase probably because endogenous TLR4 ligands produced upon inflammation and MMP-mediated cartilage degradation affect both macrophage and antigen-specific T cell responses. The high position of TLR4 pathway in the hierarchy of proinflammatory processes in arthritis provides an interesting therapeutic target for RA.

## 075 RENAL GRAFT SURVIVAL AND PATIENT MORTALITY IN WEGENER'S GRANULOMATOSIS: A CASE/CONTROL STUDY

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**Purpose:** Kidney transplantation is an important medical advance for patients with end stage renal disease (ESRD) due to Wegener's granulomatosis (WG). Small cohort studies, case reports, and pooled analyses have reported excellent graft survival rates. This study used data from the United States Renal Data System (USRDS) to compare

renal graft survival and patient mortality rates in patients transplanted for WG to patients transplanted for other causes of ESRD.

**Methods:** Cases included in this study received a renal transplant for WG between 1988 and 2004 with controls being defined as patients transplanted for causes other than WG. Cases were matched to two controls by gender, race, age at transplant (2 years), year of transplant (2 years), allograft type, and total number of transplants. Only 1st episode transplants were analyzed. Log-rank tests and Kaplan-Meier curves were used to compare renal graft survival and patient mortality rates between the two groups. For graft survival, patients were censored if documented as having a functioning graft at the time of death or if reported as lost to follow-up.  $\chi^2$  and Student's t-test were used where appropriate.

**Results:** A total of 712 WG patients were matched to 1424 controls. Mean age at transplant for both groups was 44.3 (SD 16.4,  $p = 1.0$ ) years, with 53% of both groups receiving a cadaver graft ( $p = 1.0$ ). 158 (22%) grafts failed in patients transplanted for WG compared to 366 (26%) failed grafts in transplanted controls ( $p = 0.04$ ). Chronic rejection was the most common cause of graft failure in both groups with recurrent disease occurring in 4.4% of both groups. The 6 month, 1 year, and 5 year allograft survival rates for WG were 94.4% (95% CI 92.4–95.8), 93.8% (91.8–95.3), and 86.9% (84.2–89.2), respectively. Similarly, the allograft survival rates for controls were 94.1 (95% CI 92.7–95.2), 92.6 (91.1–93.9), and 82.7 (80.6–84.6), respectively. A total of 137 (19%) WG patients died compared to 361 (25%) controls ( $p = 0.002$ ). Infection was reported as the most common cause of death in WG patients ( $n = 29$ , 21%). The most common cause of death in the control group was myocardial infarction or stroke, which was reported in 65 (18%) patients. Active disease as a cause of death could not be determined. The 6 month, 1 year, and 5 year patient survival rates for WG were 97.1% (95% CI 95.5–98.1), 95.9% (94.2–97.2), and 89.9% (87.4–91.9), respectively. The control group 6 month, 1 year, and 5 year survival rates were 96.7% (95% CI 95.6–97.5), 95.2% (93.9–96.2), and 85.7% (83.7–87.4), respectively.

**Conclusions:** Patients who received a renal transplant for WG had a lower rate of graft failure and mortality than patients transplanted for other causes. Graft failure due to recurrent disease was an uncommon cause of graft loss.

*The interpretation and reporting of these data are the responsibility of the authors and in no way should be seen as an official policy or interpretation of the US government.*

## 076 THE NEW INTERLEUKIN (IL)-1 FAMILY CYTOKINE IL-33 IS EXPRESSED BY HUMAN SYNOVIAL FIBROBLASTS AND ITS PRODUCTION IS INCREASED BY IL-1 $\beta$ AND TNF $\alpha$

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**Introduction:** IL-33 (or IL-1F11) was recently identified as a ligand for the orphan IL-1 family receptor T1/ST2. Upon binding to T1/ST2, IL-33 induces intracellular signals similar to those activated by IL-1. The T1/ST2 receptor is highly expressed on mast cells, which have been recognized as important mediators in the pathogenesis of arthritis. These observations suggest that, like other members of the IL-1 family, IL-33 might be involved in the disease process in joint inflammation. However, there is no information available to date concerning production of this cytokine by joint cells.

**Objective:** The aim of this study was to examine the production of IL-33 by synovial fibroblasts (SFs) and its regulation by pro-inflammatory stimuli.

**Methods:** Human SFs were stimulated with IL-1 $\beta$  (1 ng/ml), TNF $\alpha$  (10 ng/ml), or recombinant mature IL-33 (10 ng/ml). IL-33 mRNA expression was examined by quantitative real-time RT-PCR. IL-33 protein expression was examined by Western blotting. Expression of the long T1/ST2 and the soluble ST2 receptor isoforms was

examined by RT-PCR. IL-6 production was assessed in culture supernatants by ELISA.

**Results:** Human SFs constitutively expressed low levels of IL-33 mRNA and protein. IL-33 mRNA and protein expression were strongly increased after treatment of the cells with IL-1 $\beta$  and/or TNF $\alpha$ . In cell lysates, exclusively unprocessed 30kD pro-IL-33 was detected by western blotting. Human SFs constitutively expressed mRNA encoding short soluble ST2, but not the long signalling T1/ST2 isoform. Consistently, treatment with recombinant mature IL-33 enhanced neither IL-6 secretion, nor IL-33 mRNA expression in SFs.

**Conclusion:** The results of this study show that IL-33, a novel member of the IL-1 family of cytokines, is produced by SFs and that its production is increased by inflammatory cytokines. Locally produced IL-33 might thus contribute to the pathogenesis of joint inflammation, for instance by activating resident mast cells.

#### 077 PROTEIN DEACETYLASE INHIBITORS SUPPRESS INFLAMMATORY CYTOKINE PRODUCTION BY HUMAN MACROPHAGES AND INDUCE APOPTOSIS

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**Purpose:** In rheumatoid arthritis (RA), like in other inflammatory diseases, the activity of histone deacetylases (HDACs) is strongly repressed in macrophages at the site of inflammation. As histone hyperacetylation promotes inflammatory gene transcription, the reduction of HDAC activity has been proposed to contribute to RA pathology. Paradoxically, HDAC inhibitors have ameliorating effects in animal models of arthritis. The purpose of this study was to examine the effect of HDAC inhibitors on human macrophage activation and survival and to compare the influence of these compounds on healthy donor (HD) and RA macrophages.

**Methods:** HD peripheral blood (PB)-, RA PB- or synovial fluid (SF)-derived monocytes were differentiated into macrophages and then incubated with trichostatin A (TSA, HDAC inhibitor) or nicotinamide (NIC, inhibitor of non-histone deacetylases), in the absence or presence of TNF or LPS. Apoptosis was assessed by Annexin V/propidium iodide staining and FACS analysis. Macrophage IL-6, IL-8 and TNF production was measured by ELISA. Protein acetylation was determined by immunoblotting with anti-acetyl-lysine, anti-acetyl-histone 3 and anti-acetyl-histone 4 antibodies.

**Results:** Both TSA and NIC induced significant dose- and time-dependent apoptosis in human macrophages. Furthermore, the presence of proinflammatory stimuli (TNF- $\alpha$  or LPS) sensitized cells to TSA- and NIC-induced apoptosis. Macrophage treatment with the combination of both TSA and NIC revealed additive proapoptotic properties. RA SF but not PB macrophages demonstrated much higher sensitivity to TSA but not NIC. Additionally, increased sensitivity of SF macrophages to TSA but not NIC-induced apoptosis was even more pronounced in the presence of TNF- $\alpha$ . Both TSA and NIC almost completely blocked TNF- and LPS-induced IL-6 and TNF- $\alpha$  production in macrophages, at concentrations insufficient to induce apoptosis. At the same time both inhibitors had no influence on TNF- and LPS-induced IL-8 secretion by macrophages. Rapid time-dependent acetylation of non-histone proteins was detected in macrophage lysates following incubation with either TSA or NIC. At the same time TSA but not NIC time-dependently induced acetylation of histone 3 and histone 4.

**Conclusions:** Inhibition of HDAC and non-histone deacetylase activity in macrophages promotes cellular apoptosis, and blocks rather than enhances inflammatory cytokine production. RA SF macrophages display an enhanced sensitivity to TSA, suggesting class I/II HDACs as therapeutic targets in RA. Ongoing experiments are aimed at determining the molecular mechanism of TSA and NIC-induced apoptosis.

#### 078 12/15-LIPOXYGENASE LIMITS INFLAMMATION AND TISSUE DAMAGE IN MURINE INFLAMMATORY ARTHRITIS

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12/15-lipoxygenase (12/15-LO) has been implicated in the generation of both pro- and anti-inflammatory arachidonic acid-derived lipid mediators including 12-HETE, 15-HETE, 13-HODE and lipoxin A4, respectively. Nevertheless the role of this enzyme during the course of the inflammatory reaction remains elusive.

We demonstrate TNF-induced regulation of 12/15-LO expression in bone marrow-derived macrophages in vitro and enhanced expression of this enzyme in the inflamed synovial tissue of arthritis-prone TNF-transgenic (TNFtg) mice in vivo. 12/15-LO-deficient macrophages exhibit a significantly prolonged and elevated expression of pro-inflammatory cytokines such as IL-6 after TNF challenge. By using 12/15-LO-deficient mice we show that the absence of 12/15-LO dramatically aggravates the clinical course of experimental arthritis in two different murine arthritis models (TNFtg mice and K/BxN-serum transfer model). Histological analysis of inflamed joints revealed both an increase in the magnitude of the inflammatory reaction and in the area of bone erosions in 12/15-LO-deficient mice. Moreover, the absence of 12/15-LO promotes a decoupled and enhanced expression of a set of pro-inflammatory genes such as IL-1 $\beta$ , IL-6 and KC in the inflamed joints in vivo.

Together these data indicate a central involvement of 12/15-LO in the limitation of an inflammatory reaction and the associated tissue damage during chronic inflammatory disorders and points toward a pivotal role of 12/15-LO-derived lipid mediators in the resolution of inflammation.

#### 079 MANNANOSE BINDING LECTIN POLYMORPHISMS ARE NOT ASSOCIATED WITH RHEUMATOID ARTHRITIS—NO MORE DOUBT

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**Objectives:** In rheumatoid arthritis (RA) conflicting results have been described on the association between genotypes of the complement factor mannose-binding lectin (MBL) and disease susceptibility and severity. This might be due to underpowerment of previous research work and the fact that no confirmation cohorts were used. Therefore a different approach is warranted.

**Methods:** MBL2 gene polymorphisms were determined in two RA-cohorts (375 and 259 cases) and 637 controls. Considering MBL polymorphisms, cases and controls were categorized in groups of high (A), intermediate (B) and low (C) MBL production. The total sample size allows detection with an odds ratio of 1.37 ( $\alpha < 0.05$ ; 1- $\beta > 0.8$ ). Subgroup analysis for disease severity was done in RA cases: (1) with current or past use of anti-TNF therapy (n = 120) compared to 255 patients using other disease-modifying drugs (DMARDs); and (2) in which the disease activity score (DAS28) and anti-CCP, IgM-, IgG- and IgA-rheumatoid factor (RF) titers were determined (n = 135).

**Results:** There was no difference in the frequencies between MBL genotypes of RA cases and controls that are associated with high A (cases 54.4%, controls 57.0%), intermediate B (cases 28.9%, controls 27.5%) or low C (cases 16.7%, controls 15.5%) MBL production.

Furthermore, there was no association between RA-cases with current or past use of anti-TNF therapy versus RA-cases using other DMARDs and MBL groups: group A (anti-TNF 53.3%, no anti-TNF 54.9%), group B (anti-TNF 29.2%, no anti-TNF 30.2%) or group C (anti-TNF 17.5%, no anti-TNF 14.9%). Also, no associations were found between MBL genotype groups and DAS28 or the autoantibody titers of anti-CCP, IgA-, IgM- and IgG-RF.

**Conclusions:** MBL genotype groups are not associated with disease susceptibility in RA, nor are MBL genotype groups associated with disease severity in RA in this large study including a confirmation cohort. Compared to previous smaller studies these results may add to more definite conclusions.

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## 080 GENETIC SIGNATURES OF MURINE CYTOMEGALOVIRUS

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Murine cytomegalovirus (MCMV) encodes multiple products modulating host immune responses. Our laboratory and others previously demonstrated that early control of MCMV infection is provided by natural killer (NK) cells via the Ly49H activation receptor that recognizes m157, an MCMV-encoded ligand. In the absence of this receptor-ligand interaction, wild type (WT) C57BL/6 mice are unable to control the infection and succumb after 6–7 days. Interestingly, SCID (severe combined immune deficiency) mice, lacking adaptive immunity, are able to acutely control MCMV but succumb after about 3–4 weeks. MCMV isolated from these mice show enhanced virulence due to mutations in m157. M157 mutants failed to stimulate IFN- $\gamma$  expression in Ly49H+ NK cells and resulted in higher splenic titers than wild-type MCMV. Taken together, these studies indicated that mutations mediating immune evasion enabled mutated viruses to become the dominant population in vivo and displayed a viral genetic fingerprint that reflected host immune selective pressure from innate immune responses. Thus, we sought to delineate non-m157 mutations to uncover novel host innate immune control mechanisms.

To set the stage for analyzing mutations on a genomic scale, we sequenced the 230-kilobase genome of three wild-type Smith strain MCMV sib clones (MCMV-WT1, WT2, WT3) via shotgun sequencing. Interestingly, only two substitution differences were identified, consistent with a low mutation rate for DNA viruses after in vitro culture. Furthermore, BALB/c mice (lacking Ly49H) were subsequently infected with MCMV-WT1, and viruses were extracted from salivary glands on day 14 post infection. After 26X coverage of the MCMV genome via shotgun sequencing, no difference was identified between the input MCMV-WT1 and the output salivary gland viruses, supporting the hypothesis that MCMV has a low mutation rate in vivo. Reasoning that mutations mediating immune evasion become amplified after in vivo passage, we plan to delineate MCMV genetic changes on a genomic scale after in vivo passage in mice deficient in Ly49H and adaptive immunity and analyze mutations using a combined molecular biology and bioinformatics approach.

## 081 THE HUMAN AUTOANTIGEN hnRNP-A2 (RA33) ACTIVATES INNATE IMMUNITY PRESUMABLY VIA TLR7

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Heterogeneous nuclear ribonucleoprotein A2 (hnRNP-A2) is known to be a B- and T-cell autoantigen in rheumatoid arthritis<sup>1</sup> and was recently found to be among the primary inducers of autoimmunity in pristane-induced arthritis (PIA) in the DA.1F rat.<sup>2</sup> In rats with PIA, hnRNP-A2 is heavily overexpressed in the inflamed joint as revealed by

real-time PCR, immunohistochemistry and western blotting. Autoantibodies to hnRNP-A2 are present in pristane-injected rats already one week before disease onset, reaching maximum levels during the acute phase of PIA. Furthermore, also pronounced T cell reactivity to hnRNP-A2 showing a Th1-like phenotype can be observed prior to the appearance of symptoms of arthritis. Surprisingly, hnRNP-A2 is also able to stimulate non-primed peripheral blood mononuclear cells to produce inflammatory cytokines. The cytokine response was produced by monocytes/macrophages in a MyD88-dependent manner suggesting involvement of toll like receptors (TLR). Mammalian ribonucleoprotein (RNP) particles such as the spliceosomal small nuclear RNPs have already been shown to stimulate TLR7 and TLR8 as well as to activate innate immune cells.<sup>3</sup> A TLR ligand screening suggested that TLR7 might indeed also be involved in the response to hnRNP-A2 and preliminary data indicate that hnRNP-A2 associated RNAs may be able to trigger cytokine secretion from macrophages. Taken together, the early presence of humoral and cellular autoimmune responses to hnRNP-A2, its abundant expression in the inflamed joint and, importantly, its capability to activate the innate immune system suggests this autoantigen plays a major role in the pathogenesis of inflammatory arthritides.

1. Fritsch et al. *J Immunol* 2002;**169**:1068.
2. Hoffmann et al. *J Immunol* 2007;**179**:7568.
3. Vollmer et al. *J Exp Med* 2005;**202**:1575.

## 082 PRESENCE OF EXTRACELLULAR HDJ2 IN SYNOVIAL FLUIDS

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The J-protein Hdj2 (DNAJA1, Hsj2, Hsdj, dj-2) is one of ca. 40 known human Hsp40 chaperones. Together with chaperones of the HSP70 family they build up variable HSP70/J-protein chaperone machines. These machines are highly conserved in all species and present in all cellular organelles. They act not only as a “foldase” during protein synthesis and transport but also “fine tune” many physiological processes like signal transduction or apoptosis. Earlier we described an enhanced expression of Hsc70 (but not of Hsp70) and several J-proteins in synovial tissue (ST) from patients with rheumatoid arthritis (RA), but not in ST from patients with osteoarthritis (OA).<sup>1,2</sup> We now show that also Hdj2 is overexpressed in ST from patients with RA, but not from patients with OA. In addition, we collected SF from 136 patients with joint effusions: 51 patients with RA and 85 patients with reactive arthritis, psoriasis arthritis or another disease. These SFs were tested for the presence of Hdj2 using a specific monoclonal antibody (KA2A5.6) in immunoblots. We detected Hdj2 in 62.5% of all SFs, in different amounts. SFs of RA patients contained Hdj2 more frequently than SFs from patients with other diseases (72.6% vs. 56.6%), but the amounts of Hdj2 within positive SFs of both groups did not differ significantly from each other. Clinical and laboratory parameters (e.g. sex, age, duration of disease, markers of inflammation, autoantibodies, etc) are collected and compared with the presence and quantity of extracellular Hdj2.

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1. Kurzik-Dumke et al. *Arthr Rheum* 1999;**42**:210–20.
2. Schick et al. *Arthr Rheum* 2004;**50**:88–93.

## 083 DETECTION OF MEMBRANE PROTEINASE 3 EXPRESSION ON NEUTROPHILS USING A WHOLE BLOOD METHOD: INCREASED MPR3 EXPRESSION IN WEGENER'S GRANULOMATOSIS BUT NOT IN CHURG STRAUSS SYNDROME

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**Objective:** Presently, membrane proteinase 3 (mPR3) is detected by flow cytometric analysis on isolated neutrophils and a high



percentage of mPR3 neutrophils has been reported to be a risk factor for ANCA-associated vasculitis. The aim of this study was to determine whether the flow cytometric analysis of mPR3 expression on isolated neutrophils is applicable to whole blood (WB) samples by assessing the reproducibility and correlation of these two methods in healthy controls and to analyze mPR3 expression on neutrophils in ANCA-associated vasculitis and other autoimmune diseases.

**Methods:** Neutrophils from patients with generalized Wegener granulomatosis (genWG) (n = 53), localized WG (locWG) (n = 9), Churg Strauss syndrome (CSS) (n = 15), systemic lupus erythematosus (SLE) (n = 15), rheumatoid arthritis (RA) (n = 22), and healthy controls (n = 30) were analyzed. mPR3 and CD63 expression on surface of neutrophils were assessed by flow cytometric analysis on isolated neutrophils and whole blood.

**Results:** The percentage and median fluorescence intensity (MFI) ratio of mPR3 expression on isolated neutrophils and whole blood method correlated significantly. Three patterns of neutrophil mPR3 expression: monomodal low, bimodal, and monomodal high were identified with both methods. Both in genWG and SLE, the percentages and MFI ratios of mPR3+ neutrophils were significantly increased compared with healthy controls (p = 0.001, p = 0.000, and p = 0.038, p = 0.019, respectively).

**Conclusion:** The WB method described in this report to quantify mPR3 on neutrophils is fast, reproducible and correlates excellently with isolated neutrophils method. High mPR3 expression on neutrophils is associated with inflammatory environment and is not specific for ANCA-associated vasculitis.

#### 084 ACTIVATION OF INFLAMMATION, COAGULATION AND FIBRINOLYSIS IN PATIENTS WITH RHEUMATOID ARTHRITIS: INHIBITION BY TUMOUR NECROSIS FACTOR ALPHA BLOCKADE

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**Objective:** Rheumatoid arthritis (RA) is associated with increased cardiovascular risk and activation of inflammation and coagulation pathways. Its treatment with infliximab, a chimeric monoclonal antibody to tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), reduces inflammation, but its effects on coagulation and fibrinolysis are unknown. We therefore investigated plasma biomarkers of inflammation, coagulation and fibrinolysis before and after infliximab treatment in RA patients.

**Methods:** We studied 18 patients with active RA and 36 healthy controls. RA patients, receiving a stable dose of methotrexate (10 mg/week), were treated with infliximab (3 mg/kg) at week 0, 2, 6 and 14. At baseline and at week 14, we determined: disease activity score (DAS28), visual analogue scale (VAS) pain, erythrocyte sedimentation rate (ESR), and plasma levels of C-reactive protein (CRP), TNF- $\alpha$ , IL-6, prothrombin fragment 1+2 (F1+2) and D-dimer. In six patients, we also evaluated inflammation and coagulation parameters one hour after infliximab infusion.

**Results:** At baseline, ESR, CRP, TNF- $\alpha$  and IL-6 levels were significantly higher in the RA patients (p = 0.001–p = 0.0001), as were F1+2 and D-dimer levels (p = 0.0001). After 14 weeks of infliximab treatment, there was a significant clinical improvement (decrease in DAS28, VAS pain, number of swollen and tender joints) and a significant decrease in ESR and CRP, IL-6, F1+2 and D-dimer levels (p = 0.03–p = 0.003). The levels of TNF- $\alpha$ , IL-6, F1+2 and D-dimer significantly decreased one hour after infliximab infusion (p = 0.05–p = 0.008).

**Conclusions:** In RA patients, infliximab leads to a rapid clinical improvement and a decrease in inflammation and coagulation biomarkers. The reduction in the latter suggests that it may reduce thrombotic risk.

#### 085 A FUNCTIONAL POLYMORPHISM OF TIR-DOMAIN-CONTAINING ADAPTOR PROTEIN IS NOT ASSOCIATED WITH AXIAL SPONDYLOARTHRITIS

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**Objective:** A genetic variant of the TLR2/4 adaptor protein TIRAP (SNPC539T) was identified in a UK and in several African populations. The heterozygous genotype of this SNP has been associated with protection from severe infections. This allele results in an attenuated response to bacterial pathogens. As an exaggerated innate immune response to pathogens has been implicated in spondyloarthritis (SpA) pathogenesis, we analyzed if the heterozygous C/T genotype was under-represented in axial SpA compared to healthy controls.

**Methods:** 204 axial SpA patients and 175 population-matched controls were included. SNP C539T was determined with a sequence specific PCR and direct sequencing.

**Results:** The frequency of the haplotypes was similar in cases and controls (87% for C and 13% for T in both groups). The C/T genotype, which attenuates TLR signalling, was not under-represented in cases versus controls (19% in controls versus 24% in cases, p = 0.44). The T/T genotype was slightly lower in cases than in controls, although this was not significant (3.4% in controls versus 1% in cases, p = 0.15). Within the cases, there were no differences in disease phenotype or activity between patients with the C/C or C/T genotype.

**Conclusion:** This study did not show significant association of SNP S180L of the TLR2/4 adaptor protein TIRAP with axial SpA.

#### Endothelium and cell recruitment, extracellular matrix and fibroblasts

##### Oral presentation

#### 086 EXPRESSION OF THE ALPHA 7 NICOTINIC ACETYLCHOLINE RECEPTOR ON FIBROBLAST-LIKE SYNOVIOCYTES FROM RHEUMATOID ARTHRITIS PATIENTS: A POSSIBLE LINK BETWEEN THE NEUROLOGICAL SYSTEM AND INFLAMMATION

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**Background:** Fibroblast-like-synoviocytes (FLS) play a key role in synovial inflammation and joint destruction in patients with rheumatoid arthritis (RA). We hypothesized that the alpha7 subunit of the nicotinic acetylcholine receptor ( $\alpha$ 7nAChR), which is an essential regulator of inflammation, could be involved in FLS activation, thereby providing a link between the neurological system and the inflammatory process in the inflamed joint.

**Objective:** To determine the functional expression of the  $\alpha$ 7nAChR subunit by FLS of RA patients and to investigate the effect of specific  $\alpha$ 7nAChR agonists on pro-inflammatory cytokine production.

**Methods:** FLS were derived from arthroscopic synovial biopsies of RA patients (n = 3), and used from passage 3 to 10. The expression of  $\alpha$ 7nAChR was analyzed by both flow cytometry and immunofluorescence microscopy using fluorescein isothiocyanate (FITC)-labelled  $\alpha$ -bungarotoxin, which binds specifically to  $\alpha$ 7nAChR. In addition,  $\alpha$ 7nAChR protein was immunoprecipitated from FLS lysates using monospecific antibodies and protein A Sepharose, and analyzed by SDS-PAGE. As a positive control we used human brain and SK-N-SH cells, which are known to express  $\alpha$ 7nAChR. The results were also confirmed by reverse transcriptase-PCR (RT-PCR) with  $\alpha$ 7 specific primers. Finally, to study whether the  $\alpha$ 7 subunit may regulate cholinergic inhibition of pro-inflammatory cytokines,

we cultured FLS in the presence of TNF $\alpha$  (10 ng/ml) for 4 hours with or without nicotine or the specific  $\alpha 7$ nAChR agonist AR-R17779 (1 nM–1 mM). Concentrations of IL-6 in the culture supernatants were determined by ELISA.

**Results:** Flow cytometry showed binding of FITC-labelled  $\alpha$ -bungarotoxin to the FLS surface (8%). After overnight TNF $\alpha$  stimulation (10 ng/ml) we found significantly increased  $\alpha 7$ nAChR expression up to 22%. Immunofluorescence microscopy confirmed binding of FITC-labelled  $\alpha$ -bungarotoxin to FLS. Western blotting revealed protein expression of  $\alpha 7$ nAChR by a protein band of 35 kDa, similar to the  $\alpha 7$  subunit isolated from human brain and SK-N-SH cells. Gene expression of  $\alpha 7$ nAChR was shown by RT-PCR. TNF $\alpha$ -induced IL-6 production by FLS was significantly reduced by nicotine (100 nM–10  $\mu$ M) and AR-R17779 (1  $\mu$ M–1 mM) ( $p < 0.05$ ).

**Conclusions:** These data provide the first evidence that FLS express the nicotinic acetylcholine receptor  $\alpha 7$  subunit on the cell surface. Activation of this subunit leads to a reduction in pro-inflammatory cytokine production. Thus, these data suggest a clear link between the neurological system and FLS function. Based on these insights, further research could result in completely new therapeutic approaches for the treatment of RA.

#### 087 UPREGULATED EXPRESSION OF THE NOVEL SUMO SPECIFIC PROTEASE SENP7 IN RA SYNOVIAL FIBROBLASTS AND TNF DEPENDENT ANIMAL MODELS OF RA

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Small ubiquitin-like modifiers (SUMOs) are key post-translational regulators of a large number of proteins that play important roles in diverse cellular processes. Increased SUMOylation contributes to the activation of rheumatoid arthritis synovial fibroblasts (RASf) but the question of how SUMO-1 is regulated in RASf remains unclear. SENP7 is the most recently described family member of SUMO-specific proteases (SENP7s) that can process SUMOs to their mature forms and remove SUMO moieties from its substrates, thereby balancing SUMOylation.

Based on most recent data on the expression and function of SUMO-1 in RASf, we analyzed the expression pattern of SENP7 in RA and OA as well as in TNF-dependent animal models of RA.

Synovial tissue samples were obtained from nine RA and OA patients and used for histological analysis as well as for the isolation of synovial fibroblasts. Using specific antibodies, the expression of SENP7 was analyzed by immunohistochemistry in all synovial tissue specimens. The expression levels of SENP7 in RASf, OASF and mice synovial fibroblasts were compared by PCR and Western-blot analysis. The subcellular localization of SENP7 was studied by immunocytochemical co-staining with cell-compartment specific markers and confocal laser scanning microscopy.

Immunohistochemistry showed a clear upregulation of SENP7 in all RA synovial tissue samples compared to OA samples. SENP7 expression was most prominently in the superficial lining layer and in the sublining; staining with the macrophage CD68 marker revealed that SENP7 was predominantly expressed in fibroblasts and around blood vessels. Cultured RASf showed a marked upregulation of SENP7 mRNA as compared to OASF. These data were confirmed by Western-blot and immunocytochemistry, where RASf showed significantly higher SENP7 protein expression levels than OASF. Both RASf and OASF showed nuclear and cytoplasmic staining of SENP7, but staining was less prominent in OASF. Endogenous nuclear SENP7 protein localized to a few prominent nuclear dots. In the cytoplasm, SENP7 was localized in the centrosome, which was identified by using the centrosome marker gamma-tubulin and in Golgi-like structures as shown by co-staining with the 58k Golgi marker. These data were confirmed in hTNFtg mice, where we found an upregulation of SENP7 mRNA in SF of

TNFtg mice compared to SF of wt mice. Using immunocytochemistry, SF of wt and TNFtg mice showed the same nuclear and cytoplasmic staining for endogenous SENP7 as human SF.

Our data indicate a disease-specific upregulation of SENP7 in the RA synovium and in the synovium of hTNFtg mice. The data further suggest that SENP7 is upregulated in SF during arthritis and is found mainly in the centrosome and in Golgi-like structures. Based on the established role of SUMOs in mediating the resistance of RASf against apoptosis, we hypothesize that SENP7 is a novel player contributing to specific features of activation in RASf and, thus, to the disease process of RA.

#### 088 INTERFERON- $\gamma$ MEDIATES REORGANIZATION OF FOCAL ADHESIONS IN FIBROBLAST-LIKE SYNOVIOCYTES AND INHIBITS THEIR CAPACITY TO CONTRACT EXTRACELLULAR MATRIX

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Interferon- $\gamma$  (IFN $\gamma$ ) is a pleiotropic cytokine that is expressed in the inflamed synovium of patients with rheumatoid arthritis (RA). IFN $\gamma$  is best known for its role in orchestrating immune reactions such as antigen processing and presentation and differentiation of lymphocytes toward a Th1 phenotype. Here we demonstrate that IFN $\gamma$  modulates cell-to-matrix interactions of fibroblast-like synovioocytes (FLS) that result in their decreased capacity to contract extracellular matrix (ECM).

To test the response of FLS to IFN $\gamma$  stimulation in a simplified model of the complex synovial microenvironment, we exposed three-dimensional micromass organ cultures to IFN $\gamma$ , tumour necrosis factor or control medium. In this model system, FLS establish a lining-sublining architecture after 2 to 3 weeks in culture that strikingly resembles the synovial tissue structure. When stimulated with IFN $\gamma$ , the FLS still formed a lining-sublining architecture. The diameter of the synovial organ cultures, however, was much larger and the sublining FLS were less densely packed when compared to TNF stimulated or unstimulated organ cultures. Thus, IFN $\gamma$  stimulation had a profound impact on FLS function in their ability to contract extracellular matrix. Since matrix contraction by fibroblasts is mediated by focal adhesions that are linked to the actin cytoskeleton, we analyzed the distribution and structure of these molecular complexes in cultured FLS that were exposed to IFN $\gamma$  or control medium using immunofluorescence and confocal microscopy. In IFN $\gamma$  stimulated FLS, multiple small focal adhesions were localized mainly at the margins of the cells from which thin actin fibres emanated. By contrast, in FLS exposed to control medium, thick actin stress fibres originated from few prominent focal adhesions at the cell periphery. Further, consistent with the effect of IFN $\gamma$  in other cells, IFN $\gamma$  stimulation of cultured FLS led to the activation of the STAT1 signalling pathway as demonstrated by SDS-PAGE and immunoblotting using an antibody against phosphorylated STAT1. Together, IFN $\gamma$  stimulation of FLS resulted in STAT1 phosphorylation and remodelling of focal adhesions that is critical for FLS function in organizing the extracellular matrix.

These studies suggest a role for IFN $\gamma$  in the mesenchymal tissue response to inflammation and may provide insight into FLS behaviour and function in arthritis, especially rheumatoid arthritis.

#### 089 DISEASE-SPECIFIC UPREGULATION OF FIBROBLAST ACTIVATION PROTEIN ALPHA IN RHEUMATOID ARTHRITIS

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In rheumatoid arthritis (RA), synovitis and local bone erosion are common features caused by proteolytic and migratory activities of synovial cells. Fibroblast activation protein alpha (FAP $\alpha$ ) acts as a

dual-specificity dipeptidyl peptidase and collagenase in vitro and has been implicated in cell migration and invasion.

Based on previous data on the expression of FAP $\alpha$  in RA synovial tissue and in collagen-induced arthritis, we studied the cell-specific expression and function of FAP $\alpha$  in human RA and in a TNF $\alpha$ -dependent murine model of RA-like disease.

FAP $\alpha$  expression was studied by immunohistochemistry of synovial tissues from patients with RA and OA and from arthritic human TNF transgenic (hTNFtg) mice. In addition, expression and regulation of FAP mRNA was assessed by RT-PCR in cultured RA and OA synovial fibroblasts (RASf, OASf), normal osteoblasts, and cells of the haematopoietic lineage, including macrophages and osteoclasts. Osteoclast precursors and osteoclasts were generated from normal PBMCs by 25 ng/ml MCSF and 50 ng/ml RANKL for 16 and 21 days, respectively. TRAP staining was used to evaluate differentiation of cells. Collagenolytic activity of cells was evaluated by immunoblotting of degraded collagen type I after 16 hours.

High expression of FAP $\alpha$  was found in synovial tissues obtained from RA patients, while only weak staining, predominantly of the lining layer, was observed in OA synovial tissues. Interestingly, analyses of joints from hTNFtg mice revealed high expression of FAP $\alpha$  not only in synovial fibroblast and osteoblasts, but also by bone-resorbing osteoclasts. Moreover, transwell-cocultures of osteoclast precursors with RASf, OASf, and normal osteoblasts demonstrated the induction of FAP $\alpha$  expression in preosteoclasts exclusively by RASf. In addition, degradation of collagen type I by FAP-expressing osteoclast precursors was observed despite the presence of EDTA, suggesting an MMP-independent mechanism.

Our data suggest a disease-specific upregulation of FAP $\alpha$  in the human and mice arthritic joint. Further, the data indicate a paracrine regulation of FAP $\alpha$  expression in preosteoclasts by RASf.

#### 090 BLOCKADE OF CADHERIN-11 ON HUMAN FIBROBLAST-LIKE SYNOVIOCYTES INHIBITS MATRIX METALLOPROTEINASE ACTIVITY

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**Objective:** Cadherin-11 is a cell-to-cell adhesion molecule expressed on fibroblast-like synoviocytes (FLS) that is important in synovial development and pathology. The synovial lining in mice lacking functional cadherin-11 has a hypoplastic appearance. When challenged with arthritis, cadherin-11-deficient mice develop less inflammation than wildtype controls. In addition, arthritic joints in cadherin-11-deficient mice are uniquely protected from cartilage erosion, even in the presence of inflammation, suggesting that cadherin-11 is an important modulator of FLS behaviour. Cadherin-11-deficient FLS cell lines invaded less efficiently through a basement membrane-like substrate compared to wildtype. Invasion of FLS through substrates such as cartilage requires production and activation of matrix metalloproteinases (MMPs) to degrade the extracellular matrix. The aim of this study was to determine whether blockade of cadherin-11 on human rheumatoid arthritis (RA) FLS cell lines decreased the ability of these cells to generate enzymatically active MMPs.

**Methods:** To test this hypothesis, human RA FLS were first incubated in the presence or absence of an anti-cadherin-11 monoclonal antibody and then allowed to invade through the basement-membrane like substrate Matrigel<sup>TM</sup>. MMP functional activity was then assessed fluorometrically in invasion assay culture media using a commercially available generic MMP substrate.

**Results:** Treatment of invading human RA FLS with an anti-cadherin-11 monoclonal antibody reduced the amount of functional MMP activity released into the culture media compared to cells incubated with an appropriate isotype control.

**Conclusions:** These findings suggest cadherin-11 may alter the ability of FLS to produce enzymatically active MMPs, providing a

possible mechanism to explain why arthritic joints from cadherin-11-deficient mice are uniquely protected from cartilage erosion. This study suggests further avenues of investigation to explore regulation of MMP activity, an important mediator of joint damage in inflammatory arthritis.

#### 091 TNF BLOCKADE INCREASES LYMPHANGIOGENESIS IN MURINE AND HUMAN ARTHRITIC JOINTS

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**Objective:** To investigate the presence and regulation of lymphatic vessels in inflamed joints of mice with experimental arthritis as well as patients with rheumatoid arthritis (RA) and spondyloarthritis (SpA).

**Methods:** Lymphatic vessels and blood vessels were assessed in synovial tissue of human tumour necrosis factor transgenic (TNFtg) mice and synovial biopsies from patients with RA and SpA by immunohistochemistry for podoplanin and CD31, respectively. Assessments were performed before and after TNF blockade in all biopsies.

**Results:** Lymphatic vessels were abundantly present in the synovial tissue of hTNFtg mice as well as RA and SpA patients. The number of lymphatic vessels was positively related to the severity of synovial inflammation. Treatment with infliximab led to an increase in formation of lymphatic vessels in murine and human inflammatory tissue.

**Conclusion:** This study shows that TNF blockade promotes the proliferation of lymphatic vessels in the inflamed synovium of RA and SpA. This finding leads to the assumption that promotion of lymphangiogenesis may play an important role in efflux of cells and fluid out of the inflamed tissue.

#### 092 INVESTIGATING THE ROLE OF AUTOTAXIN AND LYSOPHOSPHATIDIC ACID (LPA) SIGNALLING IN THE PATHOGENESIS OF RHEUMATOID ARTHRITIS

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Rheumatoid arthritis (RA) is a chronic inflammatory disease, characterized by prolonged inflammation and synovial hyperplasia eventually leading to bone and cartilage destruction. Although its aetiology and pathogenesis remain poorly understood, a variety of mechanisms have been implicated in the initiation and perpetuation of synovial inflammation, including persistence of cytokine networks and other pro-inflammatory factors. Under the influence of the pro-inflammatory milieu, synovial fibroblasts (SFs) in RA become activated and hyperplastic, while releasing a number of effector signals. Activated RA SFs were recently shown to have altered actin dynamics, correlating with increased proliferation and adhesion to the extracellular matrix (ECM).

Lysophosphatidic acid (LPA) is a bioactive phospholipid that acts via specific G-protein-coupled receptors on the cell surface to activate a great variety of signalling pathways, inducing cytoskeletal reorganization, proliferation and migration in many cell types. Extracellular LPA is mainly produced by Autotaxin (ATX/Enpp2), a secreted lysophospholipase D highly expressed in a variety of tumours. In this report we show that ATX mRNA is highly expressed in mouse arthritic joints as detected with expression profiling and verified with Real-Time PCR. Moreover, massive ATX expression was detected with immunocytochemistry in arthritic joints both in animal models and human patients.

To explore a possible role of ATX and related LPA signalling in the pathogenesis of RA, we conditionally ablated ATX expression in



synovial fibroblasts and chondrocytes and assessed disease development in a well established transgenic animal model of RA. The lack of ATX expression in the joints resulted in marked decreased inflammation and synovial hyperplasia, suggesting a possible involvement of LPA signalling in the pathogenesis of the disease. To dissect the cellular and molecular effects of LPA in synovial fibroblasts a series of ex vivo experiments were performed. TNF, the major pro-inflammatory cytokine driving RA development, was shown to induce ATX secretion from primary SFs, while LPA was shown to stimulate SFs proliferation and adhesion, most likely through rearrangement of their actin cytoskeleton. Moreover, the LPA effect was shown to be dependent on the activation of a number of cellular signalling pathways that have been reported to contribute to RA pathogenesis, including ERK, JNK and p38 activation. Together the above results suggest an important role of ATX and LPA signalling in RA pathogenesis.

### 093 EFFECTS OF INTERFERON $\beta$ ON NEOANGIOGENESIS IN ARTHRITIS

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Improved understanding of the pathophysiology of rheumatoid arthritis (RA) has led to the identification of a large number of potential therapeutic targets. As neovascularization is one of the early changes in rheumatoid synovial tissue (ST) and appears to be necessary for pannus formation, the use of anti-angiogenic agents to prevent vascularization of inflamed joints could be a novel therapeutic approach. Interferon (IFN)- $\beta$  is a type I IFN with pleiotropic function. It has recently been shown that activated osteoclasts and stromal cells, including macrophages and fibroblasts, are responsible for exacerbated CIA in IFN- $\beta$  deficient mice. This implies that IFN- $\beta$  is involved in regulating the activation state of these cells. In addition, IFN- $\beta$  exerts a variety of anti-inflammatory and chondroprotective effects in various animal models of rheumatoid arthritis (RA). The aim of the present study is to investigate the effect of IFN- $\beta$  on neoangiogenesis in animal models of arthritis and in patients with RA.

An adenoviral vector encoding the IFN- $\beta$  gene (AAV-IFN- $\beta$ ) was used to study the effect of intra-articular IFN- $\beta$  treatment on the angiogenesis promoting factors vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2) in adjuvant arthritis (AA) in rats. The effects of daily, exogenous IFN- $\beta$  therapy on neoangiogenesis were evaluated in the collagen-induced arthritis (CIA) model in mice. Synovial tissue of the hind paws was examined by immunohistochemistry. In addition, ELISA was used to measure VEGF secretion by IL-1 $\beta$  stimulated RA fibroblast-like synoviocytes (FLS) cultured in the presence of IFN- $\beta$ . Finally, the in vivo effect of systemic IFN- $\beta$  treatment on angiogenesis was examined in serial synovial tissue (ST) samples from RA patients by immunohistologic examination and digital image analysis.

The study revealed that intra-articular adenoviral gene therapy, leading to continuous IFN- $\beta$  expression in the synovium, resulted in a decrease in VEGF and FGF-2 expression in the ST of rats with AA. Accordingly, exogenous IFN- $\beta$  treatment reduced the number of newly formed blood vessels in mice with CIA significantly. Consistent with these data, IL-1 $\beta$  induced VEGF production by RA FLS in culture could be completely blocked by exogenous IFN- $\beta$ . Systemic treatment of RA patients with IFN- $\beta$  revealed an inhibitory effect on angiogenesis. In contrast to the experience in the animal models, this was not associated with robust clinical effects.

These data suggest that IFN- $\beta$  has a direct effect on the production of angiogenesis promoting factors and is a potent negative regulator of angiogenesis in RA. In contrast to observations in rodent models, inhibition of neoangiogenesis appears not sufficient to induce clinically meaningful improvement in RA.

### 094 INFLUENCE OF POTENTIAL MODULATORS LIPOXIN A4, BLACK TEA EXTRACT AND RESVERATROL ON INTERLEUKIN-1 $\beta$ - AND SERUM AMYLOID A-INDUCED INFLAMMATION AND COAGULATION IN HUMAN PRIMARY ENDOTHELIAL CELLS

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**Background:** Inflammation plays a key role in the development of endothelial dysfunction and atherosclerotic/thrombotic events. However, not all types of endothelial cells have the same predisposition to develop inflammatory-driven atherosclerosis/thrombosis. Both interleukin-1 $\beta$ , as a pro-inflammatory cytokine, and serum amyloid A, as an acute phase protein, have been implicated as reactants initiating pathophysiologic processes in the endothelium. Endogenous anti-inflammatory lipid mediators (lipoxin A4) or plant extracts (black tea extract and resveratrol), could lead to the modulation of endothelial activation.

**Aim of study:** The objectives of this study were to understand the influence of potential anti-inflammatory modulators, lipoxin A4, black tea extract and resveratrol, on interleukin-1 $\beta$ - and SAA-induced human primary endothelial cells and to compare their interleukin-6 expression, NF-kappaB DNA binding, tissue factor expression and to determine the levels of released prostacyclin.

**Materials and methods:** Human primary endothelial cells derived from different origins (umbilical vein or HUVEC, coronary artery or HCAEC and microvascular HMVEC) were grown in order to determine the mRNA and protein levels of IL-6 and tissue factor by RT-PCR and ELISA. Released prostacyclin levels were determined by competitive EIA and NF-kappaB DNA binding was measured by ELISA.

**Results:** We found that IL-1 $\beta$  induction of IL-6 was dose-dependant in all three cell types at the protein as well as the mRNA levels, with the highest responses seen in HCAEC, and these cells were used in order to confirm the data with NF-kappaB DNA binding. Similarly, this had been confirmed previously for SAA-induced IL-6. Black tea extract and resveratrol inhibited IL-6 only on HCAEC and HUVEC, with little or no effect on HMVEC, where IL-1 $\beta$  or SAA stimulation was very low. There was no modulation upon addition of lipoxin A4 (at concentrations of 1 and 100 nM) to serum amyloid A and interleukin-1 $\beta$  treated cells, respectively, on either IL-6 protein or mRNA levels in any of the cell types. Comparison of tissue factor mRNA levels and tissue factor activity showed distinct cell specificity. Prostacyclin, non-enzymatic metabolite, 6-keto PGF1 $\alpha$  was elevated dose-dependently in both HCAEC and HUVEC following interleukin-1 $\beta$  and SAA cell stimulation, however levels were lower in HCAEC. Black tea extract elevated even further the interleukin-1 $\beta$ - and SAA-stimulated 6-keto PGF1 $\alpha$  release on all three cell types, while resveratrol caused a strong inhibition.

**Conclusions:** In summary, both black tea extract and resveratrol act as inhibitors of IL-6; however, they have opposing effects on prostacyclin in HUVEC and HCAEC. There are distinct significant differences in the levels of IL-6 between the various cell types, with HCAEC showing the highest responses that could account for greater susceptibility to inflammation and atherogenesis of the coronary artery.

## Cartilage and bone destruction

### Oral presentation

### 095 REGULATORY T CELLS SUPPRESS OSTEOCLAST FORMATION: A NEW LINK BETWEEN THE IMMUNE SYSTEM AND BONE

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**Introduction:** Osteoclastogenesis depends on the cellular environment provided by pre-osteoblasts or activated synovial fibroblasts. T

lymphocytes are another cell type which stimulates osteoclast formation. Osteoclast stimulation by activated TH1 cells has revolutionized the understanding of immune-mediated inflammatory bone loss observed in most chronic inflammatory diseases. Based on this observation we hypothesized that regulatory T cells (Tregs) that counteract activated T cells might exert a different effect.

**Aim:** To investigate the role of Tregs in osteoclastogenesis.

**Methods:** CD4+CD25+foxp3+ Tregs were purified from spleen and co-cultured with CD11b+ osteoclast precursor cells isolated from bone marrow. Osteoclastogenesis was assessed by TRAP staining and bone erosion by pit resorption assay. Trans-well and cytokine blocking experiments were performed to define the mechanisms of interaction between regulatory T cells and osteoclasts.

**Results:** Tregs but not activated T cells dose-dependently inhibited MCSF- and RANKL-dependent osteoclast formation and resorbing activity by up to 80%. The inhibition was not due to an alteration of RANKL/OPG balance, but required direct cell-cell contact via CTLA-4. Expression of TGF-beta, IL-4 and IL-10 by Tregs contributed to the inhibitory effect on osteoclastogenesis.

**Conclusion:** These data showing that regulatory T cells suppress osteoclast formation provide a new link between the immune system and bone and extend our knowledge on the regulation of bone homeostasis by the immune system.

## Oral presentation

### 096 THE FOUR AND A HALF LIM ONLY PROTEIN 2 (FHL2) IS INVOLVED IN THE STABLE ACTIVATION OF RHEUMATOID ARTHRITIS FIBROBLASTS THROUGH REGULATING MMP-13 EXPRESSION AND INVASIVE BEHAVIOUR

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The scaffolding four and a half LIM-domain only protein 2 (FHL2) is a mediator of protein-protein interactions and involved in the regulation of cellular processes that have been shown to be of relevance for rheumatoid arthritis. Specifically, FHL2 has been shown to interfere with mesenchymal cell activation and has been described as an interacting partner of the  $\beta$ -subunit of integrin as well as focal adhesion- and mitogen-activated protein kinases.

Here, we studied the expression of FHL2 in RA and OA synovial tissues and analyzed its expression and function in RA and OA synovial fibroblasts (RASf and OASf) as well as in histological sections obtained from the human TNF transgenic (hTNFtg) mice and from TTP-/-, both of which develop an RA-like destructive arthritis due to high levels of TNF $\alpha$ .

Using immunohistochemistry with FHL2 specific antibodies, the expression of FHL2 was studied in synovial tissue samples of RA and OA patients as well as synovial tissue samples taken from hTNFtg and TTP-/- mice. SFs were isolated from these tissues, and the expression of FHL2 was analyzed by western blot. siRNA was used to knock down FHL2-expression in RASf. FHL2 and MMP-13 expression was analyzed by western blot and real-time PCR. To investigate the influence of FHL2 on the invasive behaviour of RASf, FHL2 was silenced and the invasion was measured by a recently established TEER assay with Vitrogen 100-coating of the epithelial cell layer. The invasion assay was performed in presence and absence of TNF $\alpha$  (100 ng/ml).

We found a high expression of FHL2 in all RA synovial tissues with most prominent staining in the synovial lining and at sites of joint destruction. OA synovium exhibited only weak staining for FHL2. As seen in western blot analysis, RASf showed a prominent upregulation of FHL2 as compared to OASf. However, chronic

exposure of synovial fibroblasts to TNF $\alpha$  inhibited the expression of FHL2, and knock down of FHL2 increased the TNF $\alpha$ -induced MMP-13 expression. Analysis of fibroblast invasion revealed a higher invasiveness of TNF $\alpha$ -stimulated RASf compared to non-stimulated cells. In line with the MMP expression data, knock down of FHL2 significantly increased the invasiveness of TNF $\alpha$ -stimulated RASf as compared to controls. Analysis of FHL2 expression in histological sections from hTNFtg and TTP-/- mice showed a significantly higher expression of FHL2 early in disease, which however decreased to an intermediate level during disease progression.

Our data point to an important role for FHL2 in inhibiting the resolution of inflammatory tissue injury in RA. In line with observations in wound healing, our results suggest that the early and constant upregulation of FHL2 in RASf reflects healing attempts within the inflamed synovial tissue, while its downregulation by TNF $\alpha$  and subsequent induction of MMP-13 contribute to the chronification of disease and are involved in the stable activation of RASf.

## Oral presentation

### 097 OSTEOPOROTIC BONE STRUCTURE IN MICE LACKING SYNDECAN-4

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Members of the syndecan family of heparan sulfate proteoglycans play important roles in cell adhesion and cell communication by serving as receptors for both extracellular matrix molecules and growth factors. Syndecan-4 is ubiquitously expressed and appears to be involved in cell proliferation, differentiation, adhesion and migration. It has been demonstrated that syndecan-4 is upregulated in the dermis after injury, and syndecan-4 knockout mice show a delay in wound healing. Other studies have shown that mitogen-triggered syndecan-4 expression is an intrinsic part of the pathways subtending osteoblast proliferation and mineralization.

Based on these data, we studied the role of syndecan-4 in bone development and turnover and assessed the consequences of a loss of syndecan-4 for bone stability using 12-month old, female wild type and syndecan-4 knockout mice.

High-resolution  $\mu$ CT imaging was carried out to study the effect of syndecan-4 deficiency on bone morphology in spines. In addition, spines were embedded in methylmethacrylate to prepare longitudinal sections that were used for von Kossa staining. To determine biomechanical features, distal femurs were fixed into a fracturing apparatus while the proximal part was rotated until failure. For further functional analysis, mesenchymal progenitor cells were isolated from newborn mouse calvaria of wild type and syndecan-4 deficient mice and differentiated into osteoblasts in vitro. Development of osteoblasts and their mineralization capacity were analysed by immunohistochemical staining with antibodies against osteoblast markers as well as by von Kossa staining.

$\mu$ CT analysis demonstrated an osteoporotic bone structure in the spines of syndecan-4 deficient mice. Biomechanical testing revealed a significantly higher maximum torque in the wild type group compared to syndecan-4 knockout animals. The stiffness of the femoral bone was higher in the wild type group. These data were confirmed by histological analyses, where the loss of syndecan-4 was associated with less trabecular and mineralized bone compared to wild type. Analyses of osteoblast differentiation and function in vitro showed clear differences between wild type and syndecan-4 deficient osteoblasts.

In conclusion, our data suggest that syndecan-4 deficiency is associated with the development of an osteoporotic phenotype that results in mechanically weaker bones mainly by inhibiting

osteoblast function. These data may provide the means for developing novel treatment strategies for osteoporosis.

## Oral presentation

### 098 IL-17 UPREGULATES MMP EXPRESSION, CARTILAGE DEGRADATION AND CORRELATES WITH DISEASE SEVERITY IN INFLAMMATORY ARTHRITIS

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**Introduction:** IL-17 expression is elevated in the RA joint and correlates with markers of inflammation and cartilage destruction.

**Aim:** To explore the mechanistic role of IL-17, TNF and OSM alone and in combination on matrix turnover and cartilage degradation.

**Methods:** IL-17 was measured in paired serum/synovial fluids, synovial tissue lysates and a serum cohort of patients undergoing anti-TNF therapy by ELISA. Primary RA synovial fibroblasts (SFC), human cartilage explants and primary chondrocytes were stimulated with IL-17 (50 ng/ml), TNF $\alpha$  (10 ng/ml) and OSM (10 ng/ml) alone and combined. MMP expression in culture supernatants was measured by ELISA and Gelatin Zymography. Cartilage proteoglycan depletion was measured by Safranin-O staining.

**Results:** High IL-17 levels in synovial tissue lysates were seen with no significant difference between RA ( $9109.2 \pm 2192.2$  pg/ $\mu$ g/ $\mu$ l) and PsA tissue ( $8886.6 \pm 2609.6$  pg/ $\mu$ g/ $\mu$ l). Expression was significantly higher in both RA and PsA tissue compared to non-inflammatory OA tissue ( $253.8 \pm 253.8$  pg/ $\mu$ g/ $\mu$ l,  $p < 0.01$ ). IL-17 levels in serum was only detectable in 20% of patients with a mean of  $22.9 \pm 8.6$  pg/ml. IL-17 levels in paired synovial fluids were significantly higher ( $91.4 \pm 18.8$  pg/ml,  $p < 0.01$ ) suggesting localized IL-17 production in the joint cavity. Synovial fluid IL-17 levels correlated with CRP ( $r^2 = 0.330$ ,  $p < 0.05$ ) and disease duration ( $r^2 = 0.470$ ,  $p < 0.05$ ). Detectable IL-17 serum levels were found in 11/36 patients in a cohort undergoing anti-TNF therapy. 80% of patients showed decreased IL-17 3-months post therapy and were defined as clinical responders, while IL-17 levels increased in patients defined as non-responders.  $\delta$ IL-17 pre/post anti-TNF therapy correlated with  $\delta$ CRP ( $r^2 = 0.817$ ,  $p < 0.01$ ). IL-17 levels also correlated with 8oxo-dG ( $r^2 = 0.448$ ,  $p < 0.05$ ) a marker of oxidative damage, IL-8 ( $r^2 = 0.554$ ,  $p < 0.05$ ) and OSM ( $r^2 = 0.553$ ,  $p < 0.05$ ). In cartilage explants, primary RASFC and primary chondrocytes, IL-17 and OSM combined significantly upregulated MMP-1 production ( $p < 0.05$ ). A similar effect was seen for MMP-13 in cartilage and chondrocyte cultures ( $p < 0.05$ ). IL-17 in combination with TNF significantly upregulated the expression of MMP-1 and -13 in cartilage and chondrocytes. The combination of TNF $\alpha$  and IL-17 had no significant effect on MMP-13 production in RASFCs but potentiated MMP-1 production ( $p < 0.05$ ). IL-17 alone and combined with TNF and OSM modulated MMP-2 and -9 expression in cartilage and RASFCs. Cartilage Safranin-O staining showed minimal proteoglycan depletion in response to IL-17, TNF and OSM alone but combined resulted in near complete proteoglycan depletion.

**Conclusion:** These data demonstrate that IL-17 is produced in the joint cavity and correlates with measures of clinical disease and response to therapy. IL-17 alone and in combination with TNF and OSM regulates matrix turnover and cartilage destruction suggesting a critical role in driving the inflammatory process in arthritis.

### 099 THE "ALARMIN" S100 A8 IS A REGULATOR OF CHONDROCYTE ACTIVATION DURING EXPERIMENTAL ARTHRITIS

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**Purpose:** In a previous study we have shown that "alarmins" S100A8 and S100A9 which can form dimers and are predominantly

produced by myeloid cells are involved in MMP-mediated cartilage destruction during experimental arthritis.<sup>1</sup> S100A8 forms the active part which is stabilized by S100A9 and protects S100A8 from degradation. As MMP mediated cartilage destruction is particularly found around the chondrocyte this prompted us to investigate whether S100A8 and A9 are produced by chondrocytes and whether these "alarmins" are actively involved in MMP release and chondrocyte activation.

**Methods:** S100A8 and A9 proteins were detected in inflamed knee joints of immune complex mediated murine arthritis using immunolocalisation. Murine chondrocyte cell line H4 was stimulated with pro-inflammatory cytokines (100 ng/ml) to investigate rS100A8 and A9 production or by recombinant S100A8 (0.2, 1 and 5  $\mu$ g/ml) to investigate MMP and cytokine production. mRNA and protein levels were measured using RT-PCR and blot analysis. S100A8/A9 dimers and cytokines were measured in culture supernatant using ELISA and Luminex. Breakdown of aggrecan on the pericellular surface of the chondrocyte was measured using VDIPEN and NITEGE (MMP and aggrecanase neoepitopes) antibodies and FACS analysis.

**Results:** Immunolocalisation of inflamed knee joints depicted that S100A8 and S100A9 proteins were abundantly expressed in chondrocytes. Expression was particularly found in the superficial layers of the cartilage surfaces at the margins of the joint. Stimulation of murine chondrocytes by pro-inflammatory cytokines IL-17, IL-18 and IFN $\gamma$  caused strong upregulation of particularly S100A8 and to a lesser extent S100A9 mRNA (S100A8: 24, 48 and 4 fold and S100A9: 4, 4 and 0 fold, respectively). Stimulation of chondrocytes by rS100A8 caused a significant autoinduction of S100A8 and to a lesser extent upregulation of S100A9 mRNA and protein levels. High concentrations of IL-6 was measured in the culture supernatant whereas TNF $\alpha$ , IL-1 $\alpha$  and  $\beta$  were below detection level. Moreover MMPs (-2, -3, -9, -13) and ADAMTS (-4, -5) mRNA levels in the chondrocyte were strongly upregulated (maximal at 1  $\mu$ g/ml (4, 4, 3, 16, 8 and 4 times, respectively)). VDIPEN and NITEGE neoepitopes on the pericellular membrane of chondrocytes were significantly elevated after stimulation with rS100A8 for 24 hours in a concentration (0.2, 1 and 5  $\mu$ g/ml) dependent manner (VDIPEN 17%, 67%, 108% and NITEGE 8%, 33% and 67%, respectively).

**Conclusions:** The alarmin S100A8 is produced by chondrocytes and directly activates MMP and aggrecanase mediated pericellular matrix degradation. S100A8 may be an important mediator of cartilage destruction.

1. Myeloid related proteins S100A8/S100A9 regulate joint inflammation and cartilage destruction during antigen-induced arthritis. *Ann Rheum Dis* (in press).

### 100 SYNDECAN-4 REGULATES CYTOKINE-DEPENDENT MMP-PRODUCTION IN RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS AND MEDIATES JOINT DESTRUCTION IN TNF-DEPENDENT MOUSE MODELS OF ARTHRITIS

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Recently syndecan-4, a member of a new family of transmembrane heparansulfate proteoglycans, has been implicated in cytokine dependent signal transduction, matrix turnover and cell differentiation.

Here, we analysed the expression and cytokine dependent regulation of syndecan-4 in synovial fibroblasts of patients with rheumatoid arthritis (RASF) and osteoarthritis (OASF) and its influence on the expression of matrix metalloproteinases (MMP). Furthermore, we studied the function of syndecan-4 during TNF $\alpha$



mediated chronic destructive arthritis in human transgenic (hTNFtg) and TTP<sup>-/-</sup> mice.

The expression of syndecan-4 and its regulation by TNF $\alpha$  was studied by immunohistochemistry and quantitative PCR in synovial tissues and fibroblasts from RA and OA patients as well as in tissues and fibroblasts of arthritic hTNFtg and TTP<sup>-/-</sup> mice. The role of syndecan-4 in the regulation of MMP-1 and MMP-3 synthesis was analysed by ELISA following siRNA-mediated knockdown of syndecan-4 in RASF stimulated with TNF $\alpha$  and IL-1. The involved signalling pathways were studied by phospho-MARK array and Western-Blot analysis with phospho-specific antibodies against different MAPKs. The influence of syndecan-4 deficiency on the development of a TNF $\alpha$ -dependent destructive arthritis was analysed clinically and by morphometric analysis of tissue sections in hTNFtg mice that were crossed with syndecan-4<sup>-/-</sup> mice.

High expression of syndecan-4 was found in synovial tissues of all RA patients as well as in the synovia of hTNFtg and TTP<sup>-/-</sup> mice, while only weak staining was seen in human OA tissues and in wildtype animals. Isolated RASF exhibited a 4-fold higher expression of syndecan-4 than OASF, and stimulation with TNF $\alpha$  resulted in a 17-fold upregulation of syndecan-4 in RASF. These data were confirmed in SF from hTNFtg and TTP<sup>-/-</sup> mice, which exhibited a 36-fold increase in syndecan-4 expression versus their wild-type controls. siRNA-mediated knockdown of syndecan-4 in RASF resulted in a significant downregulation of TNF $\alpha$  and IL-1 induced phosphorylation of ERK and subsequently in the production of MMP-1 and -3. Knockout of syndecan-4 in hTNFtg mice resulted in a significant reduction of swollen paws and grip strength, and morphometric analysis of hTNFtg/syndecan-4<sup>-/-</sup> mice showed a significant reduction of inflammation and cartilage destruction.

Our data suggest a disease-specific and stable upregulation of syndecan-4 in RASF that triggers the activation of ERK and subsequently induces MMP-1 and -3 production. The loss of syndecan-4 protects against inflammation and cartilage destruction during TNF $\alpha$ -induced chronic arthritis in vivo.

# 101 SYNDECAN-4 IS INVOLVED IN ADAMTS-5 ACTIVATION AND AGGREGAN DEGRADATION IN OSTEOARTHRITIC CARTILAGE

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Cartilage break down by loss of proteoglycans is one of the hallmarks in osteoarthritis (OA). The aggrecanase ADAMTS-5 has been shown to be responsible for proteoglycan loss in arthritic mouse cartilage. However, the mechanisms that lead to ADAMTS-5 activation during cartilage remodelling are poorly understood.

Based on recent data that have implicated transmembrane heparan sulfate proteoglycans in matrix turnover, we analyzed the relationship between syndecan-4 and ADAMTS-5 in osteoarthritic cartilage and in different animal models of OA.

To study syndecan-4 in OA, we compared its expression in normal and OA articular cartilage by Northern-blot analysis and immunohistochemistry. For functional analysis, osteoarthritic changes were induced in syndecan-4<sup>-/-</sup> mice and their wild type controls by surgically achieved joint instability, and safranin-orange staining assessed the loss of proteoglycans. Staining for syndecan-4 and ADAMTS generated aggrecan neo-epitopes was performed in the knees of these mice. In addition, expression of syndecan-4 as well as ADAMTS generated aggrecan neo-epitopes was studied in an exercise-induced Wistar-rat model of OA. For in vitro studies proteoglycan loss was induced by IL-1 in femur cartilage caps isolated from 3 weeks old wild type and syndecan-4<sup>-/-</sup> mice. IL-1 dependent ADAMTS-5 expression was analyzed by real time PCR in chondrocytes isolated from these femur caps. ADAMTS activity

and protein content was measured in the supernatant of IL-1 treated wild type and syndecan-4<sup>-/-</sup> femur caps.

There was a strong upregulation of syndecan-4 in human OA cartilage and OA rats. Analysis of osteoarthritic changes in mice revealed a strong and early induction of syndecan-4, and there was a significant reduction of proteoglycan loss in the syndecan4<sup>-/-</sup> mice compared to their wild type controls. This was accompanied by a significantly reduced staining for ADAMTS generated aggrecan neo-epitopes in syndecan-4<sup>-/-</sup> mice. ADAMTS-5 expression was upregulated by IL-1 equally in wild type and syndecan-4<sup>-/-</sup> femur cap cartilage; however, proteoglycan loss was reduced significantly in syndecan-4<sup>-/-</sup> cartilage. Antibodies blocking syndecan-4 inhibited the IL-1 induced proteoglycan loss in wild type cartilage cultures. In the supernatant of syndecan-4<sup>-/-</sup> cartilage, the presence of activated ADAMTS proteases was significantly reduced compared to wild type controls. Surprisingly, the amount of total ADAMTS-5 protein were elevated in the supernatants of differentiated chondrocytes lacking syndecan-4 as compared to wild type cells.

Our data demonstrate that syndecan-4 expression is induced in human OA cartilage and in different rodent models of the disease. Furthermore, syndecan-4 is functionally involved in cartilage degradation by hypertrophic OA chondrocytes through activation of ADAMTS mediated cleavage of aggrecan. Inhibition of syndecan-4 may, therefore, constitute a promising strategy to interfere with osteoarthritic cartilage damage.

# 102 ESTABLISHMENT OF A MATRIX ASSOCIATED TRANSEPIThELIAL RESISTANCE INVASION (MATRI) ASSAY TO PRECISELY MEASURE THE INVASIVE POTENTIAL OF SYNOVIAL FIBROBLASTS

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Inflammation of the synovium in rheumatoid arthritis (RA) leads to progressive tissue invasion and destruction of cartilage and bone matrix. Synovial fibroblasts (SF) are key effector cells and exhibit features of stable cellular activation that result in their attachment to articular cartilage and deep invasion into the extracellular matrix. Quantification of the invasive potential of RASF as well as the assessment of treatment strategies with respect to matrix destruction has been a major challenge.

Here, we established an in vitro assay to determine precisely the invasive potential of SFs from patients with RA cultured on a collagen matrix. This Matrix Associated Transepithelial Resistance Invasion (MATRI) assay is based on the breakdown of the electrical resistance of a matrix coated epithelial monolayer.

Tissue samples of the synovial membrane of four patients with RA and four patients with osteoarthritis (OA) were obtained at joint replacement surgery. SF were isolated by enzymatic digestion of the synovial tissue and used in passages 4–6. The A7 melanoma cell line was used as positive and dermal fibroblasts as negative controls. All cells were cultured under standard conditions with 10% FCS. For the measurement of their invasiveness, a highly sensitive electrophysiological technique was used that is based on the measurement of the electrical resistance of a monolayer of the C7 subclone of Madin-Darby canine kidney cells (MDCK-C7). This high resistance clone was grown in MEM on the back of a 0.4-micrometer filter cup. 500000 cells of interest were seeded onto a collagen matrix consisting of 97% type I collagen and 3% type III collagen that was used to coat the top of the filter cup. The resistance across this monolayer was measured with a STX2 electrode. The invasion was recorded four times a day for a total period of five to six days. Measurement of all cells was performed in quadruplicates. Time to reach a 50% breakdown of transepithelial

resistance (tR50) was taken as a measure for the invasiveness and expressed as the invasion coefficient IV50 with  $IV50 = 1/t50$ .

As measured by our MATRI assay, RASF showed a strong invasiveness. Their invasion coefficient was  $IV50 = 0.024$ , which was close to that of A7 melanoma cells ( $IV50 = 0.065$ ) and significantly higher than the invasiveness of OASF ( $IV50 = 0.014$ ). While there was a clear inter-individual variability, the test discriminated well between RASF and OASF and showed a high reliability. Collectively, our data demonstrate that the MATRI assay constitutes a highly sensitive electrophysiological method to analyze the invasive potential of RASFs and constitutes a novel tool for analysing novel therapeutic strategies that aim at modulating the invasive potential of RASF.

### 103 EXPRESSION OF CATHEPSIN K AND MMP-1 BUT NOT MMP-3 INDICATES PERSISTENT OSTEODESTRUCTIVE ACTIVITY OF OSTEOCLASTS IN LONGSTANDING ANKYLOSING SPONDYLITIS

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**Background:** Ankylosing spondylitis (AS) is a frequent chronic rheumatic disease characterized by new bone formation in the spine. The pathogenesis, although strongly influenced by different genes, is unknown. Especially the role of persistent inflammation in the spine has remained unclear.

**Objective:** To analyze tissue specimen obtained by biopsy from predefined locations of the spine of patients with established AS undergoing anterior and posterior osteotomy of the spinal column, and to compare these findings by immunohistochemistry to specimen obtained from patients with degenerative disc disease (DDD) who also had surgical interventions at the spine.

**Material and methods:** Bony and soft tissue specimens from 30 patients with AS who underwent spinal osteotomy for correction of hyperkyphosis were obtained during the operation from the following sites: (1) articular processes, (2) spinous processes including interspinous ligaments and (3) intervertebral disks. The findings were compared to samples obtained during circumferential fusions from 20 patients with DDD. The immunohistological analysis included the detection of cathepsin K, MMP-1, MMP-3 and RANK ligand as markers of osteodestructive changes.

**Results:** Numerous cathepsin K positive multinucleated cells were found in the articular processes adjacent to fibrous tissues. In addition, smaller mononuclear cells attached to bone expressed MMP-1. In the spinous processes, the numerous cathepsin K-positive multinucleated cells were accompanied by unstained mononucleated cells. At this site, where the interspinous ligaments are attached, MMP-1 positive cells were found to invade bone. In the intervertebral disks, a large number of mononucleated cells were positive for cathepsin K. In general, much more cathepsin K or MMP-1-positive cells could be observed in AS, compared to DDD. Isolated cells expressing these matrix degrading enzymes could be found in DDD, some attached to bone, but never invading, as it was the case in AS. In contrast, no differences were found for expression of MMP-3 between AS and DDD. The expression of RANK ligand was detected only in AS patients.

**Conclusion:** Cathepsin-K, one of the main collagenolytic proteases in osteoclasts, is, unlike other proteases such as MMP-3, strongly expressed in the spine of patients with AS, clearly different to DDD. Mainly mononuclear cells, fibroblast-like cells, and cells attached to bone and sites of bone remodelling express cathepsin K assuming high osteoclastic activity in those regions. This supports the view that persistent inflammation has an important role in the pathogenesis of AS. How these osteodestructive changes relate to osteoproliferation remains to be determined.

### 104 THE ROLE OF INTERLEUKIN-1 IN TNF-MEDIATED SACROILIITIS

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**Introduction:** Sacroiliitis is the hallmark of ankylosing spondylitis (AS). Tumour necrosis factor alpha (TNF $\alpha$ ) is a major driver for sacroiliac inflammation in AS, as demonstrated by the clear clinical effect upon TNF blockade. It is, however, not clear whether TNF directly or indirectly through other cytokines, such as interleukin-1 (IL-1), causes sacroiliac inflammation.

**Methods:** We crossed mice overexpressing human TNF $\alpha$  (hTNFtg), which spontaneously develop bilateral sacroiliitis, with mice lacking IL-1 $\alpha$  and  $\beta$  (IL-1/-/-). Sacroiliac inflammation, bone and cartilage erosion, as well as osteoclast formation were histologically analyzed at three time points (8, 12 and 16 weeks of age).

**Results:** Whereas hTNFtg arthritic mice clinically deteriorated over time, IL-1 deficiency led to a significant preservation of weight and physical function in arthritic mice. Histological examinations showed no difference of inflammatory parameters and signs of joint destruction at the age of 8 and 12 weeks in hTNFtg mice, regardless of the IL-1 phenotype. In contrast, there was a highly significant decrease in inflammation ( $p < 0.001$ ), bone erosion ( $p < 0.001$ ) and osteoclast formation ( $p < 0.01$ ) at week 16 in hTNFtg mice lacking IL-1 as compared to hTNFtg mice with intact IL-1 signalling.

**Conclusion:** IL-1 deletion reduces sacroiliac inflammation and preserves structural joint integrity at late disease stages in the hTNFtg mouse. There is evidence for an important role of IL-1 in the progression of sacroiliac joint destruction.

### 105 BiP: A NEGATIVE REGULATOR OF OSTEOCLAST DIFFERENTIATION AND FUNCTION

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**Background:** Dysregulated bone remodelling is often the major component underlying the pathology of a number of human diseases, including rheumatoid arthritis. In such conditions there is an increased presence of bone resorbing osteoclasts. To combat these diseases, inhibitors of osteoclast differentiation are required. Our previous work with BiP suggests that this stress protein affects the differentiation of peripheral blood monocytes so we investigated the effect of BiP on osteoclast differentiation from monocytes and bone resorption ability.

**Methods:** Human peripheral blood monocytes were matured with macrophage-colony stimulating factor (M-CSF) and soluble ligand for receptor activator of NF- $\kappa$ B (RANKL) in the presence or absence of BiP. Osteoclast development was measured by the presence of F-actin rings and bone resorption by pit formation following culture on dentine slices and staining with toluidine blue. Additionally the ex vivo murine calvarial model of bone resorption was used.

**Results:** BiP potently inhibited the differentiation of osteoclasts in vitro when added with M-CSF and RANKL (F-actin rings: control  $39.3 \pm 13.4$ ; BiP 2  $\mu$ g/ml  $5.6 \pm 2.2$ ; BiP 20  $\mu$ g/ml  $1.7 \pm 1.5$ ; BiP 50  $\mu$ g/ml  $1.3 \pm 0.8$ ). Additionally, BiP added to semi-mature osteoclasts, after 10 days in M-CSF and RANKL, caused significant reduction in number of F-actin rings (control,  $220 \pm 48$  versus BiP,  $52 \pm 24$ ;  $p = 0.04$ ) and bone resorption dentine pits (control,  $19 \pm 7$  versus BiP 2  $\mu$ g/ml  $2 \pm 0.4$ ;  $p = 0.03$ ). The calvarial model also showed that BiP (1  $\mu$ g/ml) caused a marked reduction in calcium release from bone discs cultured with RANKL (control,  $1.75 \pm 0.2$  mg/dl versus BiP,  $0.65 \pm 0.15$  mg/dl).

**Conclusion:** The development and function of osteoclasts is inhibited by BiP and this could prove to be a novel immunotherapy for diseases with dysregulated bone remodelling.

# 106 OSTEOCLAST-ASSOCIATED RECEPTOR (OSCAR) AND ITS ROLE IN RHEUMATOID ARTHRITIS

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**Background:** Osteoclast-associated receptor (OSCAR) is a key molecule during the regulation of osteoclastogenesis. Recently the human homolog of OSCAR was characterized as a novel Fc receptor  $\gamma$ -chain associated receptor expressed by different myeloid cells, most notably by monocytes and dendritic cells. Together with an increasing number of other cell surface receptors, which are expressed on both bone-remodelling cells and immune cells, OSCAR is suggested to play an important role in both bone homeostasis and in activation of the immune system.

**Objectives:** Rheumatoid arthritis (RA) leads to severe bone and cartilage destruction. So far, little is known about the osteoclast receptors, which determine osteoclast differentiation and activity aside RANK, which are typically found during the state of immunological hyper-activation described in RA. OSCAR is a new osteoclast receptor, the function of which is unknown in RA.

**Materials and methods:** Monocytes from RA patients and healthy controls were analyzed for the expression of OSCAR by using flow cytometry (FACS). Additionally a sandwich enzyme-linked immunosorbent assay (ELISA) was established to examine the existence of soluble OSCAR in human serum. Consequently we assessed concentration levels of soluble OSCAR in serum samples from RA patients and healthy controls. Furthermore we examined bone and synovial tissue from patients with RA by means of immunohistochemistry. In addition RT-PCR analysis of monocytes from healthy volunteers was performed after stimulation with recombinant TNF- $\alpha$  and RANKL.

**Results:** Phenotypic analysis by using FACS revealed an increased expression intensity of OSCAR on the surface of monocytes from RA patients compared to healthy controls. Interestingly, expression intensity of OSCAR correlated with the severity of disease as assessed by validated and standardized clinical disease activity scores. We were able to detect soluble OSCAR in the serum of humans by creating an enzyme linked immunosorbent assay. ELISA analysis revealed a decreased concentration of soluble OSCAR in serum samples from RA patients compared to healthy controls and serum concentration inversely correlated with the DAS 28. Immunohistochemistry analysis, performed in bone and synovial tissue samples from RA patients, showed OSCAR expression in osteoclasts within inflammatory bone erosions. Moreover RT-PCR analysis showed that recombinant TNF $\alpha$  increased OSCAR mRNA expression in monocytes of healthy donors.

**Conclusion:** These data suggest an altered expression of Fc  $\gamma$ -chain associated OSCAR in rheumatoid arthritis. Increased OSCAR expression facilitates monocytes to differentiate into osteoclasts, which is a prerequisite for inflammatory bone erosion. The results of this study will contribute to a better understanding of mechanisms linking the immune system and bone homeostasis.

# 107 TUMOUR NECROSIS FACTOR ALPHA (TNF $\alpha$ )-MEDIATED INDUCTION OF MMP-3 INVOLVES PHOSPHATIDYLINOSITOL-3-KINASE GAMMA (PI3K $\gamma$ )

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The gamma isoform of the phosphatidylinositol-3-kinase (PI3K $\gamma$ ) has been implicated recently in the recruitment of inflammatory

cells during experimental arthritis, but the question of whether PI3K $\gamma$  is involved directly in joint destruction remains unclear.

Here, we analyzed the expression of the gamma isoform of PI3K in synovial fibroblasts from patient with RA (RASf) and OA (OASF). In addition, we investigated the effects of PI3K $\gamma$  on TNF $\alpha$ -induced p42/44-phosphorylation and MMP expression as well as on the invasive behaviour of RASf and synovial fibroblasts from arthritic human TNF transgenic (hTNFtg) and hTNFtg/PI3K $\gamma$ -/- mice.

RASf and OASF were analyzed for the expression of mRNA for the catalytic p110 and regulatory p101 subunit of PI3K $\gamma$  by PCR and western blot. The involvement of PI3K $\gamma$  in p42/44-phosphorylation was studied in TNF $\alpha$ -stimulated cells (100 ng/ml, 20 min) using the pan-PI3K-inhibitor LY294002 (200 nM) and a PI3K $\gamma$  specific inhibitor AS-252424 (50–100 nM). The expression of MMP-3 was studied by ELISA following stimulation of synovial fibroblasts with TNF $\alpha$  (10 ng/ml, 24 h). The invasive behaviour of TNF $\alpha$ -stimulated RASf treated with AS-252424 respectively LY294002 was investigated using a transepithelial resistant (TEER) assay. Synovial fibroblasts from hTNFtg mice crossed into PI3K $\gamma$ -/- mice were used to confirm these data in an animal model of RA.

PCR and western blot revealed the expression of p110 catalytic subunit of PI3K $\gamma$  but not of the regulatory p101 subunit in RASf. OASF showed only negligible levels of p110. Stimulation of RASf with TNF $\alpha$  resulted in the phosphorylation of p42/44 with LY294002 completely inhibiting the TNF $\alpha$ -mediated activation. AS-252424 reduced the TNF $\alpha$ -stimulated phosphorylation of p42/44 in RASf. Treatment of RASf with LY294002 and AS-252424 significantly reduced the TNF $\alpha$ -mediated induction of MMP-3 (55% and 47%, respectively). Similarly, fibroblasts from hTNFtg mice expressed significantly more MMP-3 than fibroblasts from hTNFtg/PI3K $\gamma$ -/- mice. As determined by TEER assay, AS-252424 significantly inhibited the invasive behaviour of RASf.

These data suggest a disease-specific expression of PI3K $\gamma$  in RASf that contribute to TNF $\alpha$ -mediated phosphorylation of p42/44, induction of MMP-3 and subsequently to the invasive phenotype of these cells. Therefore, specific inhibitors of PI3K $\gamma$  may be a promising strategy to interfere with the activation of RASf and to reduce cartilage destruction in RA.

# 108 PROGRESSION OF RADIOGRAPHIC DAMAGE IN PATIENTS WITH ANKYLOSING SPONDYLITIS: DEFINING THE CENTRAL ROLE OF SYNDESMOPHYTES

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**Background:** Structural spinal changes such as erosions, syndesmophytes and ankylosis are characteristic for ankylosing spondylitis (AS). These may be quantified by the modified Stokes AS spinal score (mSASSS). It is unknown which radiographic feature is most relevant for assessment of change and prediction of future damage in AS. In this study, we analysed radiographic progression in AS by using different assessment strategies and defined the most important changes and predictors of radiographic deterioration.

**Methods:** Spinal radiographs of 116 AS patients were scored by the mSASSS at baseline (BL) and after 2 years (FU). Radiographic progression was assessed by differentiating 1) any change, 2) progression to syndesmophytes/ankylosis (definite change), and 3) changes exceeding the predefined smallest detectable change (SDC). A growth angle of 45° was used to differentiate syndesmophytes from spondylophytes. Radiographic damage was defined as a scoring of  $\leq 1$  (suspicious change) and definite damage as a scoring of  $\geq 2$  (syndesmophyte).

**Results:** Novel syndesmophytes after 2 years occurred in 31% of the patients and any radiographic progression (including suspicious



changes) was detected in 42%. Thus, in 74% of the patients changes were due to syndesmophytes and/or ankylosis. In comparison, using the SDC (calculated at 2 mSASSS units), progression was seen in 28% of patients.

No radiographic damage or suspicious changes such as erosions, sclerosis or squaring (mSASSS = 1) at baseline was found in 59/116 patients (50.9%, 95% CI 41.9–59.8%). Definite radiographic progression (deterioration to syndesmophytes or ankylosis (mSASSS = 2 or 3)) after 2 years was found in 11/59 patients (18.6%, 95% CI 10.7–30.4%). In the remaining 57/116 (49.1%) patients who had definite radiographic damage already at baseline (no clinical and demographic difference to the 59 patients described above), 25 showed development of new additional syndesmophytes (43.9%, 95% CI 31.8–56.7%). Thus, in the entire group, independent of the baseline radiographic status, definite radiographic deterioration was found in 36/116 patients (31%, 95% CI 23.3–39.9%). Similar results were found when single vertebral edges were evaluated for definite progression. Furthermore, patients with definite radiographic damage at baseline had more radiographic progression than patients without radiographic damage at baseline: mean mSASSS change  $2.6 \pm 4.0$  vs.  $0.8 \pm 1.4$  ( $p = 0.002$  between groups). Using the cut-off of 45% degree growth angle, only 12% of all osteophytes appeared as spondylophytes.

**Conclusions:** Syndesmophytes and ankylosis are the most relevant structural changes in AS, also in the mSASSS. Development of just one syndesmophyte within 2 years indicates progression of structural changes in AS; this is relevant for daily clinical practice. Baseline radiographic damage is predictive for radiographic progression, and presence of syndesmophytes is most predictive.

## 109 MCP-1 DEFICIENT MICE ARE OSTEOPETROTIC

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Osteoclastogenesis is a multistep event including proliferation of osteoclast progenitors (pOCs) and their differentiation into multinuclear polycaryons. Two cytokines M-CSF and RANKL are necessary for differentiation of macrophage precursors into mature osteoclasts (OCs). However, despite these two major osteoclastogenic cytokines, other cytokines and chemokines might be essential for the modulation of osteoclastogenesis. The importance of chemokines and their receptors could be recently underlined by our finding that mice lacking the chemokine (C-C) receptor 2 (CCR2) exhibit an osteopetrotic phenotype due to impaired OC development and function. Monocyte chemoattractant protein 1 (MCP-1) is a ligand of CCR2. We therefore investigated the role of MCP-1 on OC development and function.

Osteoclastogenesis could be inhibited in vitro by using anti MCP-1 antibodies. This inhibition mainly affected the fusion of pOCs as the number of TRAP positive mononuclear cells was not altered. To investigate the role of MCP-1 in vitro we analyzed the bone phenotype of MCP-1<sup>-/-</sup> mice. Numbers of OCs detected in TRAP stained sections of MCP-1<sup>-/-</sup> tibiae were also reduced. To investigate if this reduction in OC numbers had an impact on bone homeostasis, histological sections were stained according to Von Kossa to detect calcified tissue. Histomorphological analysis revealed a slight (+13%) but significant increase in bone volume in MCP-1<sup>-/-</sup> mice, indicating an osteopetrotic phenotype of these gene deficient animals. However, when compared to the amount of increased bone (+40%) in CCR2<sup>-/-</sup> mice, the effects of MCP-1 deficiency were minor. These data demonstrate that inhibition of MCP-1, one of the various ligands of CCR2, is indeed efficient, but not as efficient as inhibition of CCR2, suggesting that targeting CCR2 should be exploited preferentially than targeting MCP-1.

## 110 INHIBITION OF BONE EROSION DOES NOT PREVENT ANKYLOSIS IN A MOUSE MODEL OF SPONDYLOARTHRITIS

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**Introduction:** The relationship between inflammation, tissue destruction and new tissue formation leading to joint ankylosis, determines the severity and prognosis of patients with spondyloarthritis. Recent data in mice and men suggest that new cartilage and bone formation and subsequent joint or spine ankylosis are uncoupled from chronic inflammation. These data challenge the classical hypothesis that inflammation and tissue damage trigger an excessive repair response in spondyloarthritis. Here, we tested whether inhibition of bone erosion by targeting osteoclasts would prevent or influence joint ankylosis in a mouse model.

**Methods:** Male DBA/1 mice from different litters were caged together at the age of 8 weeks. Treatment with zoledronic acid (100 µg/kg) or placebo was started at the age of 10 weeks and administered every two weeks by intraperitoneal injection. Clinical incidence and severity of arthritis in the hind paws were evaluated twice a week until the age of 25 weeks. At this point, bone density measurements were performed, mice were sacrificed and severity of arthritis was evaluated by histology.

**Results:** Male DBA/1 mice developed a spontaneous arthritis characterized by enthesal cartilage and bone formation leading to joint ankylosis. To inhibit osteoclast activation and bone erosion at the enthesal sites, mice were treated with zoledronic acid (100 µg/kg every two weeks, from week 10 to 24). Treatment with zoledronic acid did neither affect incidence nor clinical severity of ankylosing enthesitis in male DBA/1 mice. Histomorphological analysis did not reveal any difference between treatment or placebo. The treatment was well tolerated without overt signs of toxicity. As expected, zoledronic acid significantly increased bone mineral density and content as demonstrated by dual x-ray densitometry and peripheral quantitative computed tomography.

**Conclusion:** These data suggest that bone erosion at the entheses or at the underlying bone is not necessary to trigger enthesal ankylosis. Therefore, these observations provide further support to the concept that inflammation/tissue destruction and new tissue formation in spondyloarthritis are uncoupled events and different therapeutic targets.

## 111 EVIDENCE FOR A LINK BETWEEN INFLAMMATION AND NEW BONE FORMATION IN ANKYLOSING SPONDYLITIS

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**Background:** Spinal inflammation and new bone formation are characteristic for ankylosing spondylitis (AS), detected by MRI and conventional radiography. The link between occurrence of spondylitis and syndesmophyte-formation is unclear.

**Objectives:** To investigate whether spinal inflammation precedes new bone formation in patients with AS.

**Methods:** Spinal MRIs and conventional radiographs were available from 39 AS patients treated with infliximab (n = 26) and etanercept (n = 13) at baseline (BL) and after 2 years (2y-FU). Detailed analysis based on the mSASSS was performed by concentrating on development of new syndesmophytes at vertebral edges (VE) with or without baseline inflammatory lesions.

**Results:** Overall, 922 VEs of the cervical and lumbar spine of all patients were analysed. There was no difference in the proportion of VEs with radiographic damage and VEs with (17.6%) and without (15.6%) spinal inflammation at BL. New syndesmophytes occurred significantly more often in VEs with (6.5% and 6.2%) than without (2.1% and 2.4%) inflammation as detected by STIR and T1-post-Gd sequences at BL vs. 2y-FU ( $p = 0.002$  and  $p = 0.047$ ),

respectively. Scoring radiographic progression with the original mSASSS confirmed these results (9.2% vs. 3.4% using STIR, 12.4% vs. 3.4% by T1-post-Gd ( $p = 0.001$ )) for the VEs with and without baseline spinal inflammation, respectively.

There were 149 and 93 vertebral edges with inflammation at baseline and at the 2-year follow-up for the STIR and the T1-post-Gd MRI sequence, respectively. Definite radiographic progression occurred only in those 4/93 vertebral edges (4.3%) that still had signs of inflammation after 2 years—in contrast to vertebral edges without inflammation where no syndesmophytes developed.

**Conclusions:** Even in patients treated with anti-TNF $\alpha$ , new bone formation occurred almost 3-fold more often in regions with MRI-proven spinal inflammation at baseline. This clearly suggests a link between inflammation and radiographic progression and argues against major uncoupling of these features, as recently proposed. More effective suppression of spinal inflammation may further improve inhibition of radiographic progression in AS.

## 112 INVOLVEMENT OF THE PLA2 PATHWAY IN THE DIFFERENTIATION, FROM MONOCYTES, AND “TRANSDIFFERENTIATION”, FROM DENDRITIC CELLS, INTO OSTEOCLASTS

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**Purpose:** Joint inflammation in patients suffering from rheumatoid arthritis (RA) is characterized by the invasion of synovium by T and B lymphocytes as well as cells from the myeloid lineage such as monocytes (Mo), macrophages, and dendritic cells (DC). Bone complications of RA consist of juxta-articular erosions. The RANKL/RANK/OPG pathway controls osteoclast (OC) differentiation. OC are cellular effectors of bone resorption both in physiological and pathological conditions. They are multinucleated cells resulting from fusion of mononuclear cells from the monocytic lineage in the presence of M-CSF and RANKL which are synthesized by stromal cells. It has been proposed that DC derived from monocytes also contribute directly to the formation of OC through a pathway named “transdifferentiation” which has been demonstrated in vitro. Phospholipases A2 hydrolyze phospholipids, giving fatty acids and lysophosphatidylcholine. Fatty acids such as arachidonic acid are metabolized into mediators of inflammation such as prostaglandins.

**Methods:** Human Mo from peripheral blood, as well as DC derived from Mo using GM-CSF and IL-4, were cultured in the presence of M-CSF and RANKL. Differentiation from Mo and transdifferentiation from DC into OC was examined in the presence of specific inhibitors of the PLA2 pathway. Cytosolic PLA2 $\alpha$  activation was examined by examining Ser505 phosphorylation.

**Results:** Differentiation into OC and bone resorption, both from Mo and DC, were strongly inhibited by inhibitors of COX-1+2 (Indomethacin) and COX-2 (NS398). Phosphorylation of cPLA2 $\alpha$  was induced by incubation of Mo and DC in the presence of M-CSF and RANKL.

**Conclusion:** In our model, PLA2 pathways are involved in the differentiation and transdifferentiation into OC.

## 113 THE NATURAL COURSE OF RADIOGRAPHIC PROGRESSION IN ANKYLOSING SPONDYLITIS: EVIDENCE FOR NON-LINEAR PROGRESSION IN A LARGE PROPORTION OF PATIENTS

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**Objective:** To define the natural course of radiographic progression in ankylosing spondylitis (AS).

**Methods:** Overall, 146 AS patients who fulfilled the 1984 New York criteria and had not received anti-TNF therapy were retrospectively included. The main inclusion criterion was availability of complete sets of cervical and lumbar radiographs of at least 2 time points within 6 years. Using the best available scoring system (mSASSS), we defined the velocity of radiographic progression on this basis and in relation to the development of new syndesmophytes over years (y).

**Results:** The mean follow-up time (FU) was  $3.8 \pm 1.7$  years (1-6 y), the mean number of consecutive x-rays was 2.7 (2-6) per patient. The mean radiographic progression for the entire cohort was  $1.3 \pm 2.5$  mSASSS units/year. Any progression was found in 79% and definite progression in 60.3% patients. Thus, 76% of the progression was due to syndesmophytes and ankylosis. The progression rate was not linear, since 42.9% of patients showed a 4-fold higher progression than the mean rate and 22.5% of patients had no progression. As cut-off for defining “fast progression” we calculated a change  $>5$  mSASSS units or  $>2$  new syndesmophytes within 2 years, for “moderate progression” a change of 2.0–5.0 mSASSS units or  $<2$  new syndesmophytes, and for “slow progression” a change of  $<2$  mSASSS units or no more than 1 new syndesmophyte within 2 years. The only predictive factor for distinction between slow and fast progression was the occurrence of syndesmophytes/ankylosis at baseline (BL). Overall, there were no differences in progression rates between CS and LS. Minor changes at BL were mainly erosions (60%). Syndesmophytes at the posterior vertebral edges were seen in 45% of patients, and in 37% of those they were not combined with anterior syndesmophytes, adding to 50% of the total syndesmophyte amount for description of status.

**Conclusions:** The radiographic progression in this cohort was not linear in many AS patients. Fast or slow radiographic progression can be retrospectively identified on an individual level by depiction of new syndesmophytes or mSASSS units. There were no differences in the progression rates between CS and LS. Prediction of fast progression is only related on the occurrence and amount of AS-specific changes, such as syndesmophytes or ankylosis. Structural changes at the posterior vertebral edges are frequently not assessed but are common in AS patients and give additional information.

## 114 TREATMENT WITH 5-AZA-DEOXY-CYTIDINE IN HUMAN ARTHRITIC CARTILAGE CELLS INCREASES THE GENE EXPRESSION OF MMP-13, BMP-1, CHONDROMODULIN-1 AND ELK-1

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**Background:** The most common eukaryotic DNA modification is methylation. DNA hypomethylation is associated with gene reactivation and chromosomal instabilities. Hypermethylation in the promoter regions of the genes, on the other hand, is involved in gene repression. In osteoarthritis (OA) the matrix metalloproteinases (MMPs) are important for collagen degradation and are suspects in the development of this disease. Especially MMP-13 is present in cartilage and thought to be one of the major collagen proteinases.

BMP-2 (bone morphogenic protein 2) is an important player in chondrocyte growth and differentiation. Chondromodulin-1 is a cartilage matrix protein and known as an angiogenic inhibitor. Elk-1, a transcriptional factor, seems to enhance MMP-13 activity. Regulation by methylation in arthritic cartilage remains unknown.

**Objective:** The aim of this study was to evaluate the influence of demethylation on the gene expression of MMP-13, BMP-2, chondromodulin-1 and Elk-1 in human arthritic cartilage cells.

**Methods:** We collected eight probes of human cartilage before undergoing total knee joint replacement under sterile conditions from osteoarthritis patients. After digestion, the cells were spread out and half of them were treated with 10  $\mu$ M of demethylation agent 5-AZA-deoxy-cytidine over a period of six days. After harvesting the cells, RNA was extracted using the Trizol method and cDNA was transcribed. Gene expression for MMP-13, BMP-2,

chondromodulin-1 and Elk-1 was performed with the Taqman Realtime PCR Assay using standardized primers.

**Results:** Interestingly there was a significant increase (4-fold) of the gene expression of MMP-13. A 1.5-fold increase was demonstrated in the BMP-2, chondromodulin-1 and Elk-1 gene expression after treatment with 5-AZA-deoxy-cytidine in the human arthrotic cartilage cell cultures compared to the untreated controls.

**Conclusion:** Further investigations are needed to show if methylation plays a major role in regulating this pro-arthrotic enzyme. If so this may lead us to new therapeutic aspects of OA in the future.

## Animal models

### Oral presentation

#### 115 THE ROLE OF PTEN IN OSTEOCLAST BIOLOGY AND SYSTEMIC BONE LOSS

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Pten is a lipid phosphatase, whose substrate is phosphatidylinositol 3,4,5-trisphosphate. Therefore pten is one of the main antagonists of the PI3-kinase, which plays a major role in many important cellular functions, such as proliferation, migration or response to inflammatory stimuli.

It has been shown, that pten deficiency in osteoblasts results in increased bone formation and osteopetrosis. Because pharmacological blockade of pten would affect osteoblasts and osteoclasts, both cell types critically involved in bone homeostasis, we investigated the role of pten in osteoclast biology.

Here we show that in vitro osteoclastogenesis in mice with a conditional, monocyte/macrophage-specific pten knock-out (LysMCrePtenflox/-) is increased by a factor of two compared to control wild-type mice. This phenotype was also detectable in vivo, where analysis of tibiae of female mice revealed a significant increase in numbers of osteoclasts per bone perimeter (N.Oc/B.Pm) and osteoclast surface per bone surface (Oc.S/BS). However, enhanced osteoclastogenesis did not result in systemic bone loss, since no differences in bone volume per tissue volume (bv/tv), trabecular thickness and trabecular separation could be detected. Although in LysMCrePtenflox/- mice only the monocyte/macrophage lineage but not mesenchymal cells including osteoblasts are affected, osteoblast activity as indicated by increased mineral apposition rates was enhanced, whereas osteoblast numbers were not different. These data show that pten deficiency in osteoclasts and their precursors leads to increased numbers of osteoclasts, which, however, seem to be functionally impaired. Interestingly, pten-/- osteoclasts have a positive regulatory role on osteoblast function, as evidenced by an increased mineral apposition rate.

Therefore in a therapeutic setting of osteoporosis, pharmacological inhibition of pten in osteoblasts and osteoclasts could lead to a powerful direct and indirect stimulation of osteoblasts with subsequent stimulation of bone formation.

### Oral presentation

#### 116 THROMBIN-ACTIVATABLE CARBOXYPEPTIDASE B PREVENTS ANTI-COLLAGEN ANTIBODY INDUCED ARTHRITIS

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Thrombin-activatable carboxypeptidase B (CPB) is well established to play an anti-fibrinolytic role by removing C-terminal lysine

residues from fibrin, thereby preventing its cleavage by plasmin. Recently, C3a, C5a, thrombin-cleaved osteopontin and bradykinin have been identified as additional substrates of CPB. CPB removes arginine residues from the C-terminus of these inflammatory mediators, thereby altering the biologic functions.

We investigated the role of CPB in arthritis using proCPB deficient mice. Arthritis was induced by an intravenous injection of anti-collagen antibodies on day 0, followed by intraperitoneal injection of lipopolysaccharide on day 3. Arthritis was assessed by visual score and paw thickness. On day 10 post-injection, joints were collected for histology. Arthritis was also studied in bradykinin receptor-/- mice and C5-/- mice. As compared to controls, proCPB-/- mice exhibited significantly more severe arthritis (on day 10 arthritis score 12.3 vs. 0.4\*; paw thickness 3.02 vs. 2.21 mm\*, \*p<0.01). Histology, graded 0–4, demonstrated severe arthritis in proCPB -/- mice versus controls (synovitis 3.7 vs. 1.2\*, pannus 2.8 vs. 1.1\*, bone erosion 3.2 vs. 0.7\*, \*p<0.01). C5-/- mice were partially resistant to induction of arthritis, while bradykinin B2 receptor-/- mice exhibited a similar severity of arthritis to controls.

These results suggest that CPB plays a critical role in regulating inflammation in anti-collagen antibody induced arthritis, and that this effect could be in part mediated by its downregulation of C5a activity. Studies are underway to determine the impact of CPB on C5a, C3a, and thrombin-cleaved osteopontin in the pathogenesis of this arthritis model.

#### 117 DIFFERENT ROLE OF IL-17 AND IFN $\gamma$ IN FC $\gamma$ RECEPTOR MEDIATED CARTILAGE DESTRUCTION DURING IMMUNE COMPLEX-MEDIATED ARTHRITIS

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**Objective:** The activating Fc $\gamma$ RI and Fc $\gamma$ RIII play a crucial role in mediating severe cartilage destruction during immune complex-mediated arthritis (ICA), which is completely blocked in Fc $\gamma$ R knockout mice. Previously it was shown that the Th1 cytokine IFN $\gamma$  aggravates cartilage destruction via upregulation of activating Fc $\gamma$ Rs. Recently we found that also the Th17 cytokine interleukin-17 (IL-17) aggravates cartilage destruction in immune complex-mediated arthritis models, but the underlying mechanism remains unknown. In this study we determined the role of IL-17 in Fc $\gamma$ R-mediated cartilage destruction during ICA.

**Methods:** ICA was passively induced by lysozyme-anti-lysozyme complexes in gamma-chain-/- mice and their wild type controls. 1.10E7 pfu AdIL-17 or control vector was injected into the knee joints of naive mice, or 1 day prior to ICA induction. Total knee joints were isolated 6 days after virus injection to study inflammation and cartilage destruction (proteoglycan depletion, chondrocyte death, MMP-mediated neopeptide expression (VDIPEN), and cartilage erosion). Synovium samples were taken for RT-PCR analysis. Macrophage (RAW) and PMN (32Dcl3) cell lines were stimulated with rmIFN $\gamma$  or rmIL-17 to study Fc $\gamma$ R expression using RT-PCR and FACS analysis.

**Results:** Compared to naive knee joints injected with control vector, IL-17 overexpression led to low inflammation and mild PG depletion. Compared to the control ICA group, a significant increase in PG depletion, chondrocyte death, VDIPEN expression, and cartilage erosion was observed in the IL-17 ICA group, 5 days after arthritis induction (p<0.02). Interestingly, knee joints in the IL-17 ICA group were severely inflamed, characterized by a massive PMN influx, which was not observed in the control ICA or IFN $\gamma$  enhanced ICA group. Strikingly, no differences were found in the IL-17 ICA group with respect to the influx of macrophages. RT-PCR analysis of the synovium showed that IL-17 overexpression during arthritis clearly enhanced mRNA expression of Fc $\gamma$ RI (96 fold),



FcγRIIb (114 fold), FcγRIII (87 fold), and FcγRIV (315 fold). In vitro stimulation of macrophages and PMNs with IL-17 showed that, in contrast to IFNγ, IL-17 does not directly regulate FcγR expression. Even though inflammation in the IL-17 ICA group of gamma-chain<sup>-/-</sup> mice was similar to their wild type controls, PG depletion was significantly reduced ( $p < 0.02$ ) and severe, irreversible cartilage destruction was completely absent.

**Conclusions:** This study shows that IL-17 significantly aggravates cartilage destruction during immune complex-mediated arthritis, which is exclusively mediated by FcγRs, since severe cartilage destruction is completely absent in gamma-chain<sup>-/-</sup> mice. In contrast to IFNγ, IL-17 does not directly regulate FcγR expression on macrophages or PMNs. Instead, IL-17 enhances cartilage destruction by strongly elevating the local amount of FcγR bearing neutrophils and underlines an additional mechanism of destruction.

#### 118 BIOLOGICAL FUNCTIONS OF HEPATOCYTES AND MYELOID CELLS AS CELLULAR SOURCES OF INTERLEUKIN-1 RECEPTOR ANTAGONIST IN THE CONTROL OF SYSTEMIC OR ARTICULAR INFLAMMATORY RESPONSES

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**Background:** Interleukin-1 (IL-1) belongs to a family of cytokines playing an important role in inflammatory and autoimmune diseases. IL-1 possesses a natural inhibitor, IL-1 receptor antagonist (IL-1Ra), that interferes with the binding of IL-1 to its cell surface receptor IL-1RI. Different cell types are able to produce IL-1Ra. Hepatocytes produce IL-1Ra in large amounts in response to inflammatory stimuli as an acute-phase response, and thus may represent a major source of IL-1Ra in the circulation during systemic inflammation. In addition, at the site of inflammation, activated myeloid cells, including macrophages and neutrophils, produce large amounts of IL-1Ra, and may play a key role in controlling articular inflammation.

**Objective:** To determine the relative contribution of hepatocytes and myeloid cells versus other cellular sources of IL-1Ra in the control of inflammatory responses in vivo.

**Methods:** We have developed a mouse model (floxed Il-1rn mice), in which the Il-1rn exon 2 is flanked by loxP sites. These mice have been crossed with albumin- or lysozyme-Cre recombinase transgenic mice to generate conditional hepatocyte- or myeloid cell-specific IL-1Ra deficient mice, respectively. Genotyping was performed by quantitative PCR and Southern blot analysis. The production of IL-1Ra was determined by ELISA, Western blot and RNase protection assay.

**Results:** Circulating levels of IL-1Ra and concentrations of IL-1Ra in tissues are not modified by the presence of LoxP sites in homozygous floxed Il-1rn mice. The results of quantitative PCR and Southern blot analysis showed 65% Il-1rn exon 2 excision specifically in the liver of conditional hepatocyte-specific knockout mice. After lipopolysaccharide (LPS) challenge, IL-1Ra mRNA and protein levels were specifically decreased in the liver. In addition, the circulating levels of IL-1Ra were decreased by approximately 60% in hepatocyte-specific IL-1Ra deficient mice as compared to control mice.

**Conclusion:** The concordance between the percentage of hepatic Il-1rn disruption and the decrease in serum levels of IL-1Ra suggests that hepatocytes are indeed a major source of circulating IL-1Ra during systemic inflammation induced by LPS. As IL-1Ra deficient mice exhibit uncontrolled inflammatory responses to LPS leading to increased lethality, we propose to use hepatocyte-conditional knockout mice to determine whether the acute phase production of IL-1Ra by hepatocytes plays a protective role in LPS-induced systemic inflammation. In addition, we have also generated myeloid cell-specific IL-1Ra knockout mice in the DBA/1

background to determine the role of myeloid cells as a source of IL-1Ra in collagen-induced arthritis (CIA). The results of conditional myeloid cell-specific knockout mice will be compared to those of IL-1Ra knockout mice, known to develop early onset CIA with severe inflammation and joint damage.

#### 119 CHARACTERIZATION OF SERUM MICROVESICLES IN AGGREGAN-INDUCED AUTOIMMUNE MURINE ARTHRITIS

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Cell-derived microvesicles (exosomes, ectosomes and apoptotic bodies) are produced upon exposure to cell activation and apoptosis-inducing stimuli. Recently, these structures have been also recognized as important players in cell-cell communication.

In this study we induced experimental autoimmune aggregan arthritis (PGIA) in BALB/c mice, and investigated serum microvesicles in arthritic animals ( $n = 9$ ) and adjuvant controls ( $n = 9$ ). Serum derived microvesicles were isolated by ultracentrifugation, and the presence of microvesicles in the preparations was confirmed both by flow cytometry and transmission electron microscopy. Immune phenotyping of microvesicles has been carried out by flow cytometry, and microvesicle-associated antibodies and aggregan have been detected by ELISA.

The percentage of microvesicles positive for annexin V, CD3, B220 or CD14 did not differ significantly between aggregan arthritic and adjuvant control mice. However, acutely arthritic serum samples were characterized by significantly elevated percentages of Fc gamma RII/III positive/annexin V negative and gamma/delta TCR positive/annexin V negative microvesicles as compared to the control serum samples.

Electron microscopy of serum microvesicles, isolated by ultracentrifugation, revealed an increase in the exosomes/ectosomes ratio in arthritic mice as compared to the adjuvant controls. We found that microvesicles in the systemic circulation of aggregan-induced arthritic mice carried antibodies reactive to cartilage aggregan. Moreover, by using an aggregan-specific monoclonal antibody (BE 553, IgG), we could detect microvesicle-associated aggregan in the serum of arthritic mice.

Syngeneic non-immune mice were injected intraarticularly on days 0 and 7 with washed microvesicles from either arthritic mice or adjuvant controls ( $n = 5-5$ ). Some mice received intraarticular PBS injections only. The mice were sacrificed on day 14. Histology of the injected joints did not reveal any inflammation upon intraarticular inoculation of serum derived microvesicles.

These data suggest that T cell-, B cell- and monocyte-derived microvesicles are present in the serum; changes in their composition may reflect activation of certain cell types during arthritis. They may carry antibodies and antibody-antigen complexes possibly bound to Fc gamma receptors; however, the direct arthritogenic potential of microvesicles remains to be elucidated.

#### 120 AGGREGAN-INDUCED ARTHRITIS IN HISTIDINE DEFICIENT MICE

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Increased numbers of mast cells (MCs) are found in the synovial tissues and fluids of patients with rheumatoid arthritis (RA), and at sites of cartilage erosion. MC activation has been reported for a significant proportion of rheumatoid specimens. Because the MC contains potent mediators, including histamine, its potential contributions to the processes of inflammation and matrix degradation have recently become evident; thereafter we investigated the potential protective effect of histamine deficiency against

aggrecan-induced arthritis. To study the role of histamine in RA we investigated cartilage proteoglycan (aggrecan)-induced arthritis in histidine decarboxylase-knockout (HDC-KO) mice, with complete lack of endogenously produced histamine. Aggrecan-induced arthritis was similar in HDC-KO and WT mice. Arthritis was even more severe in HDC-KO mice than in the wild type animals 10 weeks following the immunization with aggrecan (arthritis score  $1.2 \pm 1.5$ ;  $0 \pm 0$ , respectively,  $p = 0.01$ ). At later time points arthritis scores were similar in both HDC-KO and WT mice. Since T lymphocyte dysfunction has an important role in the pathogenesis of RA, next we investigated T cell signal transduction and cytokine production in HDC-KO and WT mice. In the absence of histamine, elevated  $\text{INF}\gamma$  mRNA and protein levels of splenocytes ( $p < 0.001$ ;  $p = 0.001$ , respectively) were associated with a markedly increased (2.5 fold,  $p = 0.0009$ ) nitric oxide (NO) production, compared to wild type animals. Furthermore, histamine treatment decreased the NO production of splenocytes from both wild type and HDC-KO mice ( $p = 0.001$ ;  $p = 0.0004$ , respectively). NO precursor (Z)-1- [2-(2-aminoethyl) - N - (2-ammonioethyl) amino] diazen-1-ium-1,2-diolate-diethylenetriamine (NOC-18) elicited  $\text{INF}\gamma$  production ( $p = 0.0002$ ), suggesting the role of NO in regulating  $\text{INF}\gamma$  synthesis. Cytoplasmic  $\text{Ca}^{2+}$  concentration of unstimulated T cells and the T cell activation-induced  $\text{Ca}^{2+}$ -signal were increased in T cells from the HDC-KO mice ( $p = 0.02$ ;  $p = 0.04$ , respectively), while the T cell activation-induced CD3 internalization was similar in both HDC-KO and wild type animals. Our present data indicate that histamine deficiency does not protect against aggrecan induced arthritis. Th1 cytokine pattern and increased NO production may both contribute to the sensitivity of HDC-KO mice to aggrecan induced arthritis. Furthermore our data indicate that histamine, in addition to its direct effects on T lymphocyte function, regulates cytokine production and T cell signal transduction through regulating NO production.

## 121 TISSUE DRAINING LYMPH NODES MODIFY CELLULAR CONTENT DURING PROGRESSION OF RHEUMATOID ARTHRITIS

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**Purpose:** Collagen induced arthritis (CIA) represents a well established animal model for human rheumatoid arthritis (RA) and allows insights into autoimmune mechanism relevant to the pathogenesis of RA. In this study we set up experiments to study the role of antigen presenting cells in CIA as potential target cells for therapeutic intervention.

**Methods:** For the induction of CIA, DBA/1 mice were immunized with type II collagen (CII) and boosted after 10–14 days. Upon the onset of arthritis the animals were scored for clinical signs of arthritis including paw swelling and grip strength. Anti-CII antibody levels were determined by ELISA. At early time points (after initial immunization) and late time points (persistent clinical signs of arthritis) animals were sacrificed.

Joint sections were analyzed for histomorphological signs of cartilage and bone destruction and lymph node sections for changes in cellular content. In addition, joint-draining as well as non-draining lymph nodes (LN) were harvested, single cell suspensions were prepared and phenotypic analysis were performed by flow cytometry. The capacity of isolated B cells, dendritic cells (DC) and monocytes/macrophages (Mo) for CII presentation was analyzed in vitro.

**Results:** At the late stage of the disease >90% of animals developed clinical signs of arthritis and serum anti-CII antibodies. Histological analysis revealed extensive synovitis with massive cellular infiltration as well as articular cartilage and bone destruction. Increased proportions of MHC class II+ cells were observed at late time points in draining LN of animals with CIA as compared to non-draining

LN or controls. This was mainly due to an increase in CD19+B220+CD40+ B cells and to a lesser extent in CD11c+ DC or CD11b+ Mo. A decrease in proportions of CD3+ T cells was observed in parallel. In addition, a shift in the CD4+/CD8+ T-cell ratio towards CD4+ was observed in the CIA draining LN, more prominent in those of affected joints. Analysis of costimulatory molecule expression (CD80, CD86 and CD40) on B cells and DC revealed a decrease in % CD80+ and CD86+ B cells and DC. Isolated B cells, DC and Mo induced the proliferation of CII-specific T cells in the presence of antigen.

**Conclusion:** Our study demonstrates substantial changes in the distribution and activation of cells involved in the initiation and/or sustaining of the inflammatory response in CIA. Ongoing experiments will concentrate on the constitutive functional capacity of APC and on the migratory behaviour of distinct cell populations in CIA.

## 122 TREATMENT OF COLLAGEN INDUCED ARTHRITIS WITH DENDRITIC CELLS: INDUCTION OF Treg THROUGH A TOLEROGenic PHENOTYPE EXPRESSING IDO

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Dendritic cells are involved in tolerance induction. We previously demonstrated that unpulsed modulated DC with PAMPs (plasmid DNA or LPS) could prevent arthritis in collagen induced arthritis. The aim of this work was to further explore the mechanisms underlying this prevention. Therefore we used plasmid (semi-mature) or LPS (mature) modulated DC and intraperitoneally injected them in the collagen induced arthritis model to assay for: (1) the presence of functionally active Treg, (2) the induction of apoptosis, (3) the expression and activity of indoleamine 2–3 deoxygenase (IDO).

We assayed for functional activity of purified T CD4+ CD25+ cells extracted after DC injections in mice. We observed that these cells can inhibit CD4 naïve T cell proliferation when obtained after semi-matured or mature DC injections. Treg obtained after immature DC or without DC injection have regulatory function too, but lower. Both semi-mature and mature DC are apoptotic after a 24 hour stimulation. Only LPS-DC expressed elevated mRNA of IDO that is active and can produce kynurenine (a tryptophan metabolite).

The IDO expression could explain the better clinical efficacy of LPS-DC in preventing arthritis. Apoptosis of both plasmid and LPS DC need further experiments that are in process.

## Genetics and environmental factors

### Oral presentation

## 123 LOWER COPY NUMBER OF $\text{FC}\gamma$ RECEPTOR 3B GENE IS ASSOCIATED WITH RHEUMATOID ARTHRITIS

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**Introduction:** Single nucleotide polymorphisms (SNPs) are the most extensively studied form of genetic variation. Recently, another type of genetic variation, copy number variation (CNV), has attracted much attention not only because approximately 3000 genes in humans (around 12% of genes) are associated with CNV, but also because many of the CNVs are located in regions of complex genomic structure and this currently limits the extent to which genetic variants can be genotyped by using tagging SNPs.

A good example of such a complex region is the region of Fc gamma receptor (FcGR) genes. FcGRs are encoded on the long arm of chromosome 1q21-23. They play a critical role in immunity by linking the IgG antibody-mediated responses with cellular effector and regulatory functions of the immune system. The balance between the activating and inhibitory FcGRs determines the immune response. Successful genotyping of FcGRs SNPs is complicated as the nucleotide sequence of several FcGR genes exhibits extensive homology, resulting from duplication and recombination events that occurred in this cluster during the evolution.

Recently, it was reported that lower copy number (CN) of FcGR3B in humans was associated with glomerulonephritis in systemic lupus erythematosus.<sup>1</sup> Human FcGR3B is expressed mainly in neutrophils and is necessary for neutrophil tethering to immune complexes. It is suggested that reduced neutrophil expression of FcGR3B in patients with low FcGR3B CN may lead to reduced glomerular clearance of immune complexes.<sup>1</sup> Furthermore, CNV of FcGR2C was found to be associated with idiopathic thrombocytopenic purpura (ITP).<sup>2</sup> These findings as well as the critical role of FcGRs in immune response lead us to study the CNV of FcGR genes in rheumatoid arthritis (RA).

**Methods:** CNV was evaluated using FcGR-specific Multiplex Ligation-dependent Probe Amplification (MLPA) assay in 408 RA patients diagnosed according to the ACR criteria and 255 healthy controls; all of them are Caucasians.

**Results:** Lower CN of FcGR3B (frequency is 8.6% in RA patients) is associated with susceptibility to RA ( $p = 0.0007$ , OR = 4.55 (1.67–13.41)). Stratifying for anti-cyclic citrullinated peptide antibodies (ACPA) revealed a high association between lower CN of FcGR3B and RA both in ACPA negative and positive subsets ( $p = 0.00001$ , OR = 8.42 (2.89–25.63) vs  $p = 0.000045$ , OR = 5.19 (2.06–13.48)), respectively. Additionally, lower CN of FcGR3B was associated with early onset of bone erosions in RA ( $p = 0.014$ , OR = 2.87 (1.13–7.49)).

**Conclusion:** A lower copy number of FcGR3B predisposes to RA as well as to early erosive disease.

1. Aitman, et al. *Nature* 2006;**439**:851–5.

2. Breunis, et al. *Blood*, prepublished online 7-9-2007; DOI 10.1182/blood-2007-03-079913.

## Oral presentation

### 124 IRF5 RISK HAPLOTYPE IS ASSOCIATED WITH HIGH SERUM INTERFERON $\alpha$ ACTIVITY IN SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS

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**Objective:** A haplotype of IRF5 has been associated with risk of systemic lupus erythematosus (SLE), and previous work from our group has demonstrated that high serum interferon alpha (IFN $\alpha$ ) activity is a heritable risk factor for SLE. We hypothesize the IRF5 SLE-risk haplotype may mediate risk of SLE by predisposing to high serum IFN $\alpha$  levels.

**Methods:** IFN $\alpha$  was measured in 199 SLE patients of European and Hispanic ancestry using a sensitive functional reporter cell assay. The rs2004640, rs3807306, rs2070197, rs10488631, rs10954213 and rs2280714 SNPs in IRF5 were genotyped in the same patients. Haplotypes were categorized as SLE-risk, neutral, or protective (R, N, and P) based on published data.

**Results:** SLE patients with R/R and R/N IRF5 genotypes had higher serum IFN $\alpha$  activity than those with P/P and N/P genotypes ( $p = 0.025$ ). This differential effect of IRF5 genotype on serum IFN $\alpha$  was driven largely by SLE patients who were positive for

either anti-RNA binding protein (anti-RBP) antibodies such as anti-Ro, La, Sm, or RNP, or those positive for anti-dsDNA autoantibodies ( $p = 0.012$  for R/R or R/N vs. P/P or N/P). No difference in IFN $\alpha$  activity by IRF5 genotype could be seen in patients lacking either type of autoantibody or in patients positive for both classes of antibodies.

**Conclusions:** The IRF5 SLE-risk haplotype is associated with higher serum IFN $\alpha$  activity in SLE patients, and this effect is most prominent in patients positive for either anti-RBP or anti-dsDNA autoantibodies. This study provides biologic relevance at the protein level for the SLE-risk haplotype of IRF5.

## Oral presentation

### 125 HLA-DRB1\*01 AND HLA-DRB1\*04 MICROCHIMERISM IN WOMEN WITH RHEUMATOID ARTHRITIS

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**Background:** Incidence of rheumatoid arthritis (RA) is increased among people who carry shared epitope (SE), a special 5 aminoacids sequence, in DR $\beta$  chains of their HLA-DRB1 molecules. However, around 20% of patients with RA do not have this susceptibility motif. For these patients, exposure to HLA allele carrying SE during fetal life has been suggested to play a role. The mechanism by which non-inherited HLA antigens from mother (NIMA) could confer susceptibility to RA is not known. Microchimeric cells (Mc) from mother and/or fetus that persist after pregnancy for many years have been implicated in some autoimmune diseases.

**Objective:** We studied if women with RA have an increased chance to be exposed to SE-positive HLA-DRB1 allele by maternal and/or fetal microchimeric cells. Secondly, we tested prevalence of HLA-DRB1\*01 and DRB1\*04 Mc in our group of RA women and healthy controls.

**Methods:** Among 141 HLA-DRB1 typed women with RA, for 51 we obtained also HLA-DRB1 typing of mother and/or all children. We compared them to 51 control women whose mother and/or children were HLA-DRB1 typed. Microchimerism was quantified by real-time PCR specific for HLA-DRB1\*04 and HLA-DRB1\*01. We tested 107 non-HLA-DRB1\*04 women (43 with RA and 64 controls) for HLA-DRB1\*04 Mc and 79 non-HLA-DRB1\*01 (33 with RA and 46 controls) for HLA-DRB1\*01 Mc. Mc was tested in DNA from peripheral blood mononuclear cells (PBMC).

**Results:** First, we considered women who were negative for SE and whose mothers and/or all children were HLA-DRB1 typed. Twenty-five percent (13/51) of women with RA were SE-negative women versus 80% (41/51) of control women. SE-negative women with RA had a known (maternal and/or fetal) source of SE-positive Mc cells twice more often than controls, respectively, 39% (5/13) versus 17% (7/41).

When tested for HLA-DRB1\*01 Mc, 30% of women with RA were positive versus 4% of healthy controls. Moreover, levels of HLA-DRB1\*01 Mc were higher among women with RA than in controls with respective mean values of 34 and 2 Mc cells per 1 million host PBMC.

When subjects were tested for HLA-DRB1\*04 Mc, frequencies of women with RA and controls positive for Mc were similar as above for HLA-DRB1\*01, respectively, 30% and 8%. Noteworthy, HLA-DRB1\*04 microchimerism among women with RA was very high with a mean value of 1988 Mc cells per 1 million host PBMC in comparison with 8 Mc HLA-DRB1\*04 cells in 1 million host PBMC for controls.



**Conclusion:** Our results show higher frequency of SE-positive maternal and/or fetal sources in women with RA in comparison to healthy women. HLA-DRB1\*01 and HLA-DRB1\*04 Mc is more frequent among women with RA compared to healthy women. Strikingly, Mc cells with HLA-DRB1\*04 reach very high levels of almost 0.2% of total host's peripheral blood mononuclear cells in women with RA. This is enough to influence reactivity in the immune system. We suggest that microchimeric cells possibly carrying SE may contribute to the pathogenesis of RA.

## 126 ASSOCIATION AND LINKAGE OF THE TRAF1/C5 REGION WITH SPECIFIC SUBSETS OF RHEUMATOID ARTHRITIS

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**Background:** The risk factors involved in rheumatoid arthritis (RA) result from an interplay between individual genetic background and environmental triggers. Although human leucocyte antigens (HLAs) account for ~30% of the heritable risk, the identities of non-HLA genes explaining the remainder of the genetic component are largely unknown. Based on functional data in mice, we hypothesized that the immune-related genes complement component 5 (C5) and/or TNF receptor-associated factor 1 (TRAF1), located on chromosome 9q33–34, would represent relevant candidate genes for RA. In this study we aimed to investigate whether this locus associates with RA.

**Methods and results:** By using a multi-tiered case-control study we observed significant association of SNPs located in a haplotype block that encompasses a 65 kb region including the 3' end of C5 as well as TRAF1. A sliding window analysis revealed an association peak at an intergenic region located ~10 kb from both C5 and TRAF1. This association signal, defined by SNP rs10818488, was confirmed in a total of 2719 RA patients and 1999 controls (odds ratio<sub>common</sub> 1.28, 95% confidence interval 1.17–1.39,  $p_{\text{combined}} = 1.40 \times 10^{-8}$ ) and was predominant in the RF+ACPA+ subset of RA patients. However, we also observed variability in control frequencies across populations. Compared to association studies, linkage studies have the added advantage of controlling potential differences in population structure, and are not likely to be hampered by variation in population allele frequencies. We observed evidence of linkage of the TRAF1/C5 locus by a significant overtransmission (55%) of the susceptibility allele, demonstrating departure from Mendel's law. This overtransmission was present only in the RF+ subgroup (56%) but not in the RF- patients (49%), further substantiating our initial results.

**Conclusions:** Using a candidate gene approach we have identified a novel genetic risk factor for RA. By taking advantage of two methods, namely association and linkage, we show that a polymorphism in the TRAF1/C5 region increases the susceptibility to the RF+ and/or ACPA+ subset of RA, possibly by influencing the structure or function of TRAF1 and/or C5.

## 127 WITHDRAWN

## 128 PROTECTIVE EFFECT OF NON-INHERITED MATERNAL HLA-DR ANTIGENS ON RHEUMATOID ARTHRITIS DEVELOPMENT

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Rheumatoid arthritis (RA) is a complex genetic disorder in which the human HLA-region contributes most to the genetic risk. HLA

plays a major role in the ability of the immune system to discriminate between self and non-self cells. HLA-DRB1-molecules containing the amino-acid sequence "DERAA" at position 70–74 of the DR $\beta$  chain (i.e. HLA-DRB1\*0103, \*0402, \*1102, \*1103, \*1301, \*1302 and \*1304) are less often present in patients with RA compared to healthy controls. It has been proposed that antigens transmitted from the mother, but not genetically inherited (called NIMA), can influence RA-susceptibility. Confrontation of the fetal/newborn immune system with the NIMA is supposed to have a lifelong modulating impact on the immune response of the child and thus on the chance to develop RA. Up to now, no protective NIMAs were described in autoimmunity. Therefore, we studied whether "DERAA"-containing HLA-DRB1-alleles as NIMA are associated with a protective effect.

179 families were studied, 88 from the Netherlands and 91 from the UK. The frequency of "DERAA"-containing HLA-DRB1-alleles of the Dutch mothers (16.1%), but not of the fathers (26.2%), was lower compared to the general Dutch population (29.3%;  $p = 0.02$ ). This was replicated in the English set of patients and controls ( $p = 0.01$ ). This difference could not be ascribed to a difference in "DERAA" frequency between men and women, since no differences were observed in a Dutch healthy control cohort ( $n = 420$ , OR 1.10, 95% CI 0.70–1.74;  $p = 0.65$ ). Further, of all families, 45 contained at least one "DERAA"-negative child with RA and at least one "DERAA"-positive parent. The odds for the "DERAA"-negative RA patients of having a "DERAA"-positive mother was significantly lower as compared to having a "DERAA"-positive father (OR 0.25;  $p = 0.003$ ).

These data show a protective NIMA-effect in a human autoimmune disease and indicate that in contrast to a "DERAA"-positive father, a "DERAA"-positive mother can transfer protection against RA to her "DERAA"-negative child.

## 129 LOSS OF IMPRINTING OF IGF2 CHARACTERIZES LOW INFLAMMATORY TYPE OF FIBROBLAST-LIKE SYNOVIOCYTES IN RHEUMATOID ARTHRITIS

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Heterogeneity in the inflammation status between rheumatoid arthritis (RA) synovial tissues is reflected in RA fibroblast-like synoviocytes (FLS) as a stable trait, suggesting a link between the phenotype of FLS and the inflammation status of RA tissues. FLS from high-inflammation tissues reveal a constitutive activation of transforming growth factor  $\beta$ /Activin A response program, whereas FLS derived from low-inflammation tissues are characterized by increased expression of IGF2. Normally, IGF2 is only expressed from the paternal allele in most cell types. The purpose of this study was to analyze whether the increased expression of IGF2 in RA FLS is a consequence from the loss of imprinting (LOI), i.e. transcription from both the paternal and maternal allele.

RA FLS obtained from 25 synovial tissues were cultured and genotyped for a SNP located within coding region of IGF2 gene, which can be used to discriminate between the two alleles. We identified 13 heterozygous RA FLS for the analyzed SNP. Subsequently, we quantified the relative contribution of the allelic transcripts by allele specific transcript quantification (ASTQ) in the informative RA FLS. Whereas 5 FLS showed monoallelic expression, a total of 8 FLS showed IGF2 expression from both the paternal and maternal allele, indicating LOI of IGF2. In order to establish a relationship between imprinting status of IGF2 and its gene expression levels, we measured IGF2 gene expression levels by quantitative RT-PCR. This analysis revealed that IGF2 expression was increased in the FLS that showed the disruption of IGF2 imprinting.

These findings indicate that a subset of RA FLS is characterized by high IGF2 expression as a consequence of LOI.

### 130 POLYMORPHISMS IN THE CD3Z LOCUS INFLUENCE TCR $\zeta$ EXPRESSION IN HEALTHY CONTROLS AND IN SYSTEMIC LUPUS ERYTHEMATOSUS

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**Background:** TCR $\zeta$  (CD247) functions as an amplification module in the TCR signalling cascade and is essential for assembly and surface expression of the TCR/CD3 complex. The TCR $\zeta$  chain is down-regulated in many chronic infectious and inflammatory diseases, including systemic lupus erythematosus (SLE). It is unclear whether reduced TCR $\zeta$  expression is a cause or a consequence of chronic inflammatory responses. We have addressed this question by adopting a combined genetic and functional approach.

**Methods and materials:** TCR $\zeta$  protein expression was analysed using a FACS-based expression index in 60 healthy controls and in 30 patients with SLE. A primary SNP screen suggested a link between CD3Z 3' untranslated region (UTR) polymorphism and low TCR $\zeta$  expression: we subsequently performed a more detailed analysis of allelic variation of the 3'UTR and its possible functional association with TCR $\zeta$  mRNA and protein expression. TCR $\zeta$  mRNA expression was measured by allele-specific real-time PCR and by allelic imbalance analysis. Actinomycin D chases were performed under specific inflammatory conditions (oxidative stress, hypoxia, TCR stimulation) to assess whether the observed effects were due to mRNA instability. Finally, a family-based association study in SLE assessed for disease-specific effects of the studied polymorphisms.

**Results:** There was considerable, but longitudinally stable, variation in TCR $\zeta$  expression in healthy individuals. This variation was associated with polymorphisms in the CD3Z 3'UTR in SLE patients and healthy controls. Detailed mapping of the 3'UTR revealed that the minor alleles of two SNPs in strong disequilibrium (rs1052230 and rs1052231) were the causal variants associated with low TCR $\zeta$  expression ( $p = 0.015$ ). The minor alleles of these 3'UTR SNPs were associated with one-third of the level of mRNA compared with the major allele. Actinomycin D chases suggested that inflammatory stress affected mRNA stability by further stabilising the major allele of rs1052230/rs1052231 rather than destabilising the minor allele. A family-based association analysis showed that the haplotype carrying the low-expression variants predisposes to SLE ( $p = 0.033$ ).

**Conclusions:** These results suggest that a genetically-determined reduction in TCR $\zeta$  expression, likely due to effects on mRNA stability, has functional consequences manifesting by systemic autoimmunity.

### 131 IS THERE A HIGHER EXPRESSION OF FLEDR POSITIVE ALLELES IN PATIENTS WITH DIFFUSE SCLERODERMA?

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**Background:** Scleroderma (SSc) is associated with HLA class II susceptibility alleles: HLA-DRB1\*11, DRB1\*15 and DRB1\*08. HLA-DRB1\*11 and some DRB1\*08 alleles associated with SSc encode a common "FLEDR" sequence. This 5 amino acid motif (aa positions 67–71) is in the binding groove of their DR $\beta$ 1 chains. Most studies focus on DR $\beta$ 1 chains, and do not take into account the multiple possibilities of the other DR $\beta$  chains (DR $\beta$ 3, DR $\beta$ 4, DR $\beta$ 5). Interestingly, on the HLA-DRB1\*15 haplotype, the FLEDR sequence is not on the HLA-DR $\beta$ 1\*15 chain but on the DR $\beta$ 5\*01 chain which is co-expressed.

**Objective:** In order to give a credit to the FLEDR sequence, we investigate the expression of both DRB1\*15 and DRB5\*01 in patients with SSc compared to controls.

**Methods:** Among subjects previously HLA DR typed by SSO methods, we analysed the HLA-DRB1\*15 and DRB5\*01 mRNA levels by reverse transcription real time PCR.

**Results:** Our results are very preliminary since only 13 patients and 4 controls have been tested. However, about half of the patients have a 1.5 times higher expression of HLA-DRB5\*01 compared to DRB1\*15 and they are mostly with a diffuse form of disease and with anti-topoisomerase antibodies.

**Conclusion:** In a previous HLA frequency analysis, we found a strong correlation between FLEDR positive patients and anti-topoisomerase antibodies that feature a diffuse form of SSc. In the current analysis, this subgroup of patients seems to have higher levels of DRB5\*01 (FLEDR positive) transcripts. This FLEDR motif may select specific antigens for presentation, therefore influences production of antibodies. Further studies are needed to confirm these early observations.

### 132 ASSOCIATION OF THE Hsp70 GENE LOCUS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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Systemic lupus erythematosus (SLE) is a systemic autoimmune disease of largely unknown etiology. In addition to environmental manifestation factors there is an important genetic predisposition. Multiple genetic polymorphisms may contribute to the development of lupus. Since heat shock proteins (Hsps) are important for peptide presentation of intracellular proteins, we investigated single nucleotide polymorphisms (SNPs) in the genes of several Hsps for their association with SLE. Genetic variants of Hsp70-Hom, Hsp70A1A and Hsp70A1B were analysed, which are localised in the SLE-susceptibility locus on chromosome 6p21.3. In a case-control study using a haplotype-tagging SNP approach, we identified a polymorphism in the region of Hsp70A1B, which was significantly associated ( $p = 0.0086$ ) with SLE. Evaluation of clinical data has shown that patients exhibiting the significantly associated haplotype contain higher titers of autoantibodies to dsDNA, Ro, and La. The association has been recently confirmed in an independent cohort. Functional analyses of the promoter activities of the Hsp70A1B alleles are in progress. Our results may help to gain new insights into the pathogenesis of SLE.

## Stem cells and cell therapy

### Oral presentation

### 133 THE LOSS OF CHONDROGENIC POTENTIAL OF SYNOVIAL DERIVED MESENCHYMAL STEM CELLS IN RHEUMATOID ARTHRITIS MAY BE LINKED TO INFLAMMATION DRIVEN DYSREGULATION OF SOX9 EXPRESSION

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**Background:** Mesenchymal stem cells (MSCs) are now recognised as major players in joint tissue repair. Despite their ability to repair bone, we have shown that the chondrogenic potential of rheumatoid arthritis (RA) MSC cultures is low compared to

osteoarthritis (OA).<sup>1</sup> Previously, we reported that the chondrogenic potential of synovium-derived MSCs was limited by in vivo exposure to inflammation.<sup>2</sup> In the current study we examine the expression of transcription factors and markers of chondrogenic differentiation in MSC cultures established from joints to understand at which step chondrogenesis is affected.

**Method:** Patients (RA n = 8, OA n = 4) were recruited through routine clinics. Inflammation was measured at arthroscopy using visual analogue score (VAS). Control bone marrow (BM) MSCs were derived from the posterior iliac crest of normal donors (n = 4). Standard MSC cultures were derived from synovial tissue biopsies. Real-time PCR was performed and the expression of the genes of interest was normalized to GAPDH. Statistical analysis was carried out using Spearman's rank correlation.

**Results:** The expression of the p65 subunit of NF- $\kappa$ B was used as positive control for inflammation. IL-7 and SDF-1 expression are known to be induced by pro-inflammatory cytokines. The expression of all three markers correlated with VAS. As expected, I $\kappa$ B $\alpha$  (regulator of NF- $\kappa$ B) was not related to inflammation. Next we investigated whether inflammation affected the expression of Runx2 and Sox9, transcription factors regulated during chondrogenesis.<sup>2,3</sup> Sox9 and Runx2 (not detected in all samples) expression varied greatly; Sox9 showed a direct relationship with inflammation. Overnight TNF treatment of control MSC cultures resulted in down-regulation of Sox9 expression. In synovial MSCs with minimal exposure to in vivo inflammation, this regulation was maintained; however, high exposure to inflammation resulted in up-regulation of Sox9. Opposing results were observed for TGF $\beta$ 3. Expression of Sox9 and Runx2 was measured over the normal 21 day chondrogenic period. The BM MSC culture showed normal Sox9 and Runx2 expression whereas in RA MSCs (high VAS) Sox9 expression was increased during early differentiation which subsided after 3 days; there was an immediate and sustained reduction in Runx2 expression. Collagen 2a displayed a similar pattern to Sox9 confirming that chondrogenesis is altered.

**Discussion:** The initial part of this study is consistent with inflammation exposure driving the expression of the NF- $\kappa$ B, IL-7 and SDF1 genes and also that of Sox9. In RA, despite initial high expression of Sox9, it appears that the molecular events driving differentiation towards chondrogenesis are perturbed during early differentiation resulting in a major decrease in Sox9 expression and poor quality cartilage. These findings may have important mechanistic implications for the future of repair therapies in arthritis.

1. Jones, et al. *A&R* 2004.
2. Jones, et al. *EWRR* 2006.
3. Zhou, et al. *PNAS* 2006.

### 134 THERAPEUTIC EFFICACY OF MESENCHYMAL STEM CELLS IN EXPERIMENTAL ARTHRITIS

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**Purpose:** Mesenchymal stromal cells (MSC) are multipotent adult stem cells characterized by their potential of differentiation in multiple lineages and their immunosuppressive properties. This last property has previously been shown to have therapeutical application in the prevention of graft versus host disease and experimental autoimmune encephalomyelitis, suggesting that MSC may be used in the treatment of other autoimmune inflammatory diseases, such as rheumatoid arthritis (RA). Indeed, the aim of this study was to evaluate the efficacy of murine primary MSC in the experimental model of collagen-induced arthritis (CIA).

**Methods:** The adherent bone marrow cells were isolated from DBA1 mice and immunophenotyped by flow cytometry. Their MSC phenotype was further characterized by their capacity to differentiate into 3 lineages by culture in specific

inducing conditions. Immunosuppression was evaluated in concanavalin A-induced proliferative assay and mixed lymphocyte reaction. In CIA, 10<sup>6</sup> MSC were intravenously injected at various times after collagen II immunization on day 0 and boost on day 21. Arthritis was evaluated by the measure of clinical signs (paw swelling and inflammation) and immunological parameters (dosage of collagen II-specific immunoglobulins, inflammatory cytokines and proliferation of T lymphocytes).

**Results:** Primary MSC can be reproducibly isolated from DBA/1 mice as confirmed by their phenotype, trilineage differentiation potential (chondrocyte, adipocyte, osteoblast) and immunosuppressive effect. When injected on day 18 and 24, syngeneic MSC were able to significantly decrease the incidence and clinical signs of arthritis by comparison with control arthritic mice. Analysis of the immunological parameters confirmed a decreased inflammatory response in treated mice. However, when MSC were injected after the onset of arthritis no beneficial effect was observed.

**Conclusion:** This study shows the efficacy of systemic injection of syngeneic MSC in the treatment of experimental arthritis. The evaluation of the mechanisms of immune suppression is underway.

### 135 CHARACTERIZATION OF OVINE MESENCHYMAL STEM CELLS FROM BONE MARROW: APPLICATION FOR THE DEVELOPMENT OF A CARTILAGE REPAIR MODEL

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**Introduction:** Mesenchymal stem cells (MSC) are considered suitable sources for cell-based therapies in cartilage engineering. However, their capacities to differentiate into chondrocytes and regenerate a fully functional and mature tissue need to be evaluated in a pre-clinical model. The objectives of this work was to establish a sheep model of cartilage repair and aimed at (1) characterizing the bone marrow-derived MSC and (2) evaluating their differentiation potential after implantation.

**Methods:** Bone marrow aspirates of 6 individual sheep were seeded at the initial density of 1–3  $\times$  10<sup>5</sup> mononuclear cells/cm<sup>2</sup> in alpha-MEM medium supplemented with 10% SVF. Colonies of adherent cells formed after 10–15 days and were passaged at the density of 10<sup>3</sup> cells/cm<sup>2</sup>. The differentiation potential of the cells was then evaluated. First, chondrogenesis was induced by culturing the cells in micropellets in presence of BMP-2 or TGF- $\beta$ 3 for 21 days. Osteogenesis was induced by culture at low density in various media containing or not dexamethasone, NaH<sub>2</sub>PO<sub>4</sub> or BMP-2. The expression of the differentiation markers was determined by RT-PCR analysis. In vivo, two partial-thickness lesions of 4 mm diameter were created in the inner part of the patellae of the posterior legs and lesions were left empty or filled with autologous cells alone, or in presence of chitosan powder or TGF- $\beta$ 3 embedded within a fibrin clot.

**Results:** The CFU-F potential was determined to be 1–2 cells in 10<sup>5</sup> mononuclear cells after a 14 day culture. Cell proliferation was maintained for more than 10 passages with a doubling time of 12 h during the first 3 months. The adherent cells were shown to be positive for CD44, CD105 low, vimentin, and negative for CD34 and CD45. The phenotype was stable at least until passage 5. The expression of the chondrogenic differentiation markers (type II collagen, aggrecan and biglycan) was either detected or increased after differentiation and proteoglycan secretion was assessed by safranin O staining on histological sections. Expression of osteopontin was assessed by RT-PCR and mineralization was detected by alizarin red S staining. Dexamethasone was shown to greatly enhance the mineralization capacity of the cells whereas presence of BMP-2 redirected the differentiation towards adipogenesis. Adipogenesis was confirmed by oil red O staining. In vivo, the preliminary data suggest that presence of MSC and chitosan increase the repair of the lesion. Histological analysis is underway to assess the quality of the neotissue after 3 months.



**Conclusions:** For the first time, the adherent stromal fraction of the bone marrow from sheep has been characterized in detail. This study provides evidence that these may be referred as MSC since they are phenotypically, biologically and functionally closely similar to their human or murine counterparts. The pre-clinical model of cartilage repair should prove useful to assess the regeneration potential of autologous MSC.

### 136 PERMANENT, BUT INCOMPLETE, BLOCKAGE OF VITAMIN-D RECEPTOR EXPRESSION IN HUMAN MESENCHYMAL STEM CELLS YIELDS FUNCTIONAL CHONDROCYTES FOR CARTILAGE REPLACEMENT

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Mesenchymal stem cells (MSCs) are pluripotent cells capable of being differentiated into several cellular phenotypes, such as chondrocytes. However, when engineering chondrocytes intended for ultimate stem cell based treatment of cartilage damage (due to osteoarthritis and/or osteoarthrosis), it is of importance to verify that the chondrocytes produced do not retain any osteoblast- or adipocyte-derived functions.

By computer analysis, we demonstrated that the vitamin-D receptor (VDR) putatively bound to a gene network (i.e. DLX4, MSX1, FOXO1A, SOX9, FOXO3A, HES1) is known to negatively affect chondrocyte development (GenoStem Chondrocyte-Development Affymetrix Analysis, GSCDAA). Hence, we created LV-particles expressing anti-VDR-shRNA, and showed that infected MSCs displayed a permanent reduction (by some 80%) in VDR expression (RT-PCR), as well as 1,25(OH)2D3 binding. Cells were thereafter differentiated towards osteoblasts or chondrocytes, yielding the following:

1) Osteoblast differentiation was significantly affected, as evidenced by suppression of marker genes (Cbfa1, Osterix, Coll-1, Osteocalcin, SPARC, Osteopontin), and showed marked reduction of in vitro mineralization.

2) MSCs attempted differentiated towards osteoblasts, injected into the tibial muscle of SCID mice, did not cause any visible mineralization (as evidenced by X-ray and histology).

3) Chondrogenic differentiation was not affected, as assessed by early (Day 1) up- and down-regulated genes (FOXO3A, WNT5A, SOX9, SPP1, FOXO1A, Smurf2, DKK1, Coll-1) according to the GSCDAA, and late genes (Aggrecan, Coll-2 and Coll-10), as well as histological and immunohistochemical characterization of micro-pellets.

4) Co-cultures of human osteoclasts and MSCs infected with the anti-VDR-shRNA, as well as preconditioned culture media from differentiated MSCs, did not significantly alter the anticipated pattern of osteoclast activation.

5) Engineered chondrocytes developed, to a minor extent, adipocyte characteristics, like PPAR $\gamma$  and AP2 expression (RT-PCR), as well as Oil-Red-O staining.

The knock-down of VDR expression was part of a scheme to assess micro-RNAs predicted to affect acknowledged osteoblast-inducing factors (i.e. Satb2, DLX5, Cbfa1, Osterix, SP3, VDR, LEF1, ATF4, NFATc1, RNF11, TAZ, ETS1, APC2). Using different databases (MiRNA Viewer, Sanger 3.0, Ensembl etc.) and the GSCDAA to search for micro-RNAs selectively favouring chondrogenesis, several micro-RNA species (10–20) of potential interest were selected. Then, their temporal variation as a consequence of cell manipulations was determined within the period (4–9 days), when hMSCs appear sensitive to the miRNA producing machinery. Some of these micro-RNAs corroborated the results observed with the manipulation of the VDR expression.

This cell engineering approach allows the conclusion that a major (some 80%) and permanent knock-down of the nuclear VDR

receptor in hMSCs blocks development of osteoblastic features, while functional chondrogenesis is maintained.

### 137 WITHDRAWN

### 138 TGF- $\beta$ -INDUCED EXPRESSION OF IL-6 AND IL-8 IS ENHANCED BY ADHERENCE OF SYNOVIAL FIBROBLASTS TO LAMININ-1

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**Objectives:** Elevated expression of laminin-1 (LM-111) is found in the synovial membrane of patients diagnosed with rheumatoid arthritis. In a recent study we showed that attachment of synovial fibroblasts to LM-111 in the presence of TGF- $\beta$  induced a significant production of stromelysins. Here we go on to investigate the regulation of IL-1, IL-6 and IL-8 by LM-111 and TGF- $\beta$ .

**Methods:** Synovial fibroblasts were expanded in DMEM medium, incubated on LM-111-coated flasks in presence or absence of 10 ng/mL TGF- $\beta$ . Signal transduction via cytoplasmic kinases or NF $\kappa$ B was monitored by immunoblotting. Changes in steady state mRNA levels encoding the interleukins were investigated by quantitative RT-PCR. Interleukins were screened for by a multiplexed immunoarray and quantified by ELISA. Biological activity of IL-6 and IL-8 was determined by B-cell proliferation and cell migration assays.

**Results:** Growth of fibroblasts on LM-111 in presence of TGF- $\beta$  induced statistically significant mRNA responses for IL-6 (mean 3.72-fold,  $\pm 1.6$ ,  $p < 0.003$ ) and IL-8 (mean 4.5-fold,  $\pm 1.6$ ,  $p < 0.001$ ) and in supernatants significantly elevated concentrations of IL-6 (mean  $7.8 \pm 5$  ng/mL,  $p < 0.008$ ) and even higher concentrations of IL-8 (mean 55.8 ng/mL) were detected, but IL-1 was not produced. Attachment of SF to LM-111 in presence of TGF- $\beta$  transiently activated p38MAPK, ERK and SMAD2 but failed to utilize NF $\kappa$ B.

**Conclusion:** Adherence to LM-111 in presence of TGF- $\beta$  may activate synovial fibroblasts for elevated expression of IL-6 and IL-8 and thus contribute to inflammation and infiltration of mononuclear cells independently of IL-1 $\beta$  and TNF- $\alpha$ .

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## New therapeutic approaches

### Oral presentation

### 139 AAV5-MEDIATED INTRA-ARTICULAR ADMINISTRATION OF TNF SMALL INTERFERING RNA PREVENTS PROGRESSION OF ARTHRITIS

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RNA interference (RNAi) has rapidly become a powerful tool for drug target discovery, and interest is rapidly growing for extension of its application to animal disease models. To overcome the limitations of in vivo RNAi delivery, several viral vectors are used for their efficacy to deliver short hairpin small interfering (si)RNAs (shRNAs), resulting in long term silencing. The successful advanced strategies in rheumatoid arthritis (RA) gene therapy using safe viral vectors such as adeno-associated viruses (AAVs) prompted us to determine the therapeutic potential of a recombinant AAV type 5 (rAAV5) expressing two different shRNA sequences for TNF- $\alpha$  (rAAV5-shTNF), a key pro-inflammatory cytokine in RA. The validation of rAAV5-shTNF vectors on a macrophage cell line resulted in significant

dose-dependent inhibition of TNF- $\alpha$  expression. When collagen-induced arthritic mice received a single intra-articular administration of the rAAV5-shTNF vectors ( $5 \times 10^9$  particles), disease incidence and severity were dramatically decreased in injected joints compared with a control group injected with a mock rAAV5 vector. The therapeutic effect was associated with local down-regulation of both inflammatory and autoimmune components of the disease in the rAAV5-shTNF-treated group compared with controls, as assessed by a decreased IFN $\gamma$  secretion and inhibition of the collagen-specific proliferative responses of lymphocytes from the draining lymph nodes. Quantification by real-time PCR showed high and exclusive reporter gene expression in injected joints. In conclusion, the overall disease parameters were improved compared with the empty rAAV5 controls. Our data present the first proof-of-principal for the application of an AAV5-siRNA-based therapy as a feasible local strategy for TNF blockade in RA at the post-transcriptional level.

## Oral presentation

### 140 DIFFERENCES IN THE FUNCTION AND MODE OF ACTION OF CERTOLIZUMAB PEGOL AND CONVENTIONAL ANTI-TNFS

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**Introduction:** While conventional anti-TNFs are bivalent and possess an Fc region, certolizumab pegol is the first and only univalent, Fc-free, PEGylated anti-TNF. The aim of this study was to compare the function and mode of action of certolizumab pegol with conventional anti-TNFs (infliximab, adalimumab and etanercept) in a series of in vitro and in vivo systems.

**Methods:** Four different methods were utilized to compare the properties of certolizumab pegol with conventional anti-TNFs. (a) Complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC) were examined using a cell-line with a high level of surface TNF. (b) Immune-complex formation with TNF was assessed by dynamic light scattering and stoichiometry of binding to TNF trimers was measured using isothermal calorimetry. (c) Placental transfer and accumulation in milk were investigated in rats using reagents that were homologous to either conventional anti-TNFs (ie, a complete antibody) or certolizumab pegol. (d) Accumulation of fluorescently-labelled certolizumab pegol and adalimumab in the hind paws of naïve mice and mice with ongoing collagen-induced arthritis was assessed by bio-fluorescence imaging.

**Results:** (a) Certolizumab pegol was the only anti-TNF agent tested that did not kill cells with TNF on the surface by either CDC or ADCC. (b) Adalimumab and infliximab both formed large complexes with TNF in solution and bound to 2.48 and 2.46 monomers in a trimer, respectively. Etanercept did not form large immune complexes and did not appear to cross-link trimers. Because of its univalent structure, certolizumab pegol bound to 2.9 monomers in a trimer and did not form large immune complexes. (c) During pregnancy and lactation in rats, only low levels of a PEGylated Fab' anti-rat TNF crossed the placenta or accumulated in milk. In contrast, an IgG1 version of the same antibody was present at high levels in foetal plasma and milk. (d) In a mouse model of arthritis, both certolizumab pegol and adalimumab penetrated more effectively into inflamed arthritic paws than into non-inflamed paws, but the ratio of penetration of certolizumab pegol into inflamed tissue compared to normal tissue was greater and more prolonged than for adalimumab.

**Conclusion:** The unique structure of certolizumab pegol results in some fundamentally different in vitro and in vivo functions. Because certolizumab pegol is Fc-free, it does not kill by CDC and ADCC and only low levels of a homologous reagent crosses the

placenta in rats, although these data are not available in patients who are pregnant. Due to its univalent, PEGylated structure, certolizumab pegol forms smaller complexes and has a differential accumulation in inflamed tissue over normal tissue. Such differences may result in a different mode of action compared to conventional anti-TNFs in patients.

## Oral presentation

### 141 TAILOR-MADE LOCAL GENE THERAPY FOR RHEUMATOID ARTHRITIS USING GENE EXPRESSION PROFILING-DERIVED DISEASE-REGULATED PROMOTERS

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The clinical course of rheumatoid arthritis (RA) is characterized by spontaneous remissions and exacerbations of joint inflammation. In an ideal treatment for RA, delivery of biologicals parallels this intermittent disease course. Local gene therapy might enable a fine-tuned supply of proteins by transcriptionally controlling transgenes using disease-regulated promoters. Advantages of this inventive approach would be twofold: firstly, restricting expression to arthritic conditions potentially maximizes therapeutic efficacy; secondly, shutting off expression under non-inflamed conditions minimizes the risk of harmful side effects, such as immune responses. In this study, we sought to elucidate and validate disease-regulated promoters from gene expression profiling (GEP) studies of experimental arthritis.

GEP was performed on synovial tissue from a chronic collagen-induced arthritis and relapsing streptococcal cell wall arthritis. The former model was explored for genes correlating to disease severity, the latter for genes whose expression parallels inflammatory flares. From these, we deduced serum amyloid A3 (SAA3) and interleukin-1 (IL-1) as candidates that meet the prerequisites of a disease-regulated promoter: low expression under naïve conditions, highly upregulated during flares, and expression profiles correlated to arthritis severity.

Next, these promoters were validated in vitro. For this, we generated lentiviral luciferase reporters containing the murine SAA3 (−314/+50) promoter or the hybrid interleukin-1 enhancer (−3690/−2720)/human interleukin-6 proximal (−163/+12) promoter (IL-1E/IL-6P). Both showed low basal activity and a strong response to IL-1, TNF $\alpha$  and LPS in murine 3T3 and human primary RA synovial fibroblasts. Notably, SAA3 promoter strength was tenfold higher as compared to the IL-1E/IL-6P promoter. Next, the applicability for achieving regulated expression in experimental arthritis was investigated. Viral luciferase reporters were injected intra-articularly and promoter activities under naïve and arthritic conditions were measured. Luciferase levels were low in unaffected joints. In contrast, a zymosan-induced acute joint inflammation upregulated luciferase levels forty (IL-1E/IL-6P) and eighty fold (SAA3) over basal levels. Since the absolute promoter activity of the IL-1E/IL-6P was tenfold lower compared to the SAA3 promoter, we applied the prior for tailor-made interleukin-4 (IL-4) gene therapy. Using the IL-1E/IL-6P promoter massive inflammation under naïve conditions, as observed for constitutive expression, was profoundly reduced, while the cartilage protective effect of IL-4 in arthritis was retained.

This study clearly demonstrates the usefulness of GEP for elucidating disease-regulated promoters. Both the SAA3 and IL-1E/IL-6P promoter are suited for restricted expression of biologicals and the difference in promoter strength hands valuable tools for developing gene therapy tailored to the properties of the transgene.

## 142 WITHDRAWN

## 143 A CRUCIAL ROLE FOR TNFR1 (P55) IN THE PRODUCTION OF CYTOKINES BY THE INFLAMED SYNOVIUM DURING STREPTOCOCCAL CELL WALL ARTHRITIS IN MICE

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Pro-inflammatory cytokines, such as TNF $\alpha$ , IL-1 and IL-6, are produced in the inflamed joint by the synovium and play an important role in the inflammatory process and connective tissue destruction in rheumatoid arthritis (RA). Although anti-TNF treatment has proven to be effective in RA it is unclear to what extent the effect of TNF blockade on the synovium contributes to this successful therapy. Whole genome mRNA analysis (Affymetrix microarray) of the murine synovium during streptococcal cell wall (SCW) arthritis showed that the TNF receptor-1 (TNFR1, P55) was one of the most upregulated genes in the TNF pathway. In this study we evaluated the role of this receptor in the synovium during SCW arthritis and whether the TNFR1 is an attractive candidate for treatment based on RNAi-mediated knockdown. For this, a short hairpin (sh) RNA against this murine TNFR1 (shTNFR1) was cloned in an adenoviral vector under control of the mouse U6 promoter. This viral vector was validated in vitro on NIH-3T3 fibroblasts that were stably transfected with a 3xNFkappaB luciferase reporter construct. The hairpin against TNFR1 completely inhibited TNF $\alpha$ -induced luciferase expression both at protein and mRNA level. Furthermore, TNFR1 knockdown caused a marked inhibition of LPS and IL1 $\beta$ -induced luciferase expression, indicating that both mediators caused release of TNF that subsequently activated NF- $\kappa$ B in this cell-line. Since it is known that synovial cells play an important role in producing pro-inflammatory cytokines that drive inflammation, we investigated the effect of TNFR1 downregulation in these cells. Injection of adenoviruses (10E7 pfu) encoding the shTNFR1 into the knee joint cavity resulted in downregulation of this receptor, one day thereafter. Next the same amount of these adenoviruses was injected one day before the induction of SCW arthritis. At day one of arthritis there was a significant reduction (75%) in cytokine levels of IL-1 $\beta$ , TNF $\alpha$  and IL-6 as measured by Luminex in 1 hr culture supernatants of synovial tissue explants taken from shTNFR1 treated mice. In support of this there was also a reduction of synovial mRNA levels of IL-1 $\beta$ , TNF $\alpha$ , IL-6, and NOS2. With these findings we demonstrate that RNAi-mediated knockdown of TNFR1 is feasible in the synovium and that this receptor plays an important role in mediating pro-inflammatory cytokine production by the inflamed synovium.

144 REVERSION OF AN ESTABLISHED DISEASE BY VACCINATION AGAINST TNF $\alpha$  IN HUMAN TNF $\alpha$  TRANSGENIC MICE

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**Aim of the study:** As an alternative to anti-human TNF $\alpha$  mAbs, we developed an active anti-hTNF $\alpha$  immunotherapy, based on a KLH-hTNF $\alpha$  heterocomplex (TNFK). We evaluated the effect of TNFK vaccination on established arthritis in mice and we studied the duration of the potential TNFK disease modulating activity in a long term evaluation.

**Methods:** Human TNF $\alpha$  transgenic mice (TTg) received 2 IM injections of TNFK at 15 weeks of age. A group was followed during 18 weeks; two other groups were followed during 36 weeks and received respectively an injection of PBS or a boost of TNFK, at 32 weeks of age. Control group received PBS only and was followed during 18 weeks. Neutralizing capacity of anti-hTNF $\alpha$  Abs was

measured on L929 cells. Arthritides were blindly evaluated by clinical scoring of four paws and histology at sacrifice.

**Results:** Mice immunized with TNFK after the onset of the disease showed a significant amelioration of arthritis ( $p < 0.001$  vs controls, ANOVA). At 23 weeks of age, a flare was observed in all mice immunized with TNFK, demonstrating that the effect of the immunization was transient. Only mice receiving a late boost of TNFK elicited high titers of neutralizing anti-hTNF $\alpha$  antibodies. TTg mice primed with TNFK and receiving PBS at 32 weeks of age showed a significant decrease of antibody titers, with a peak 15 weeks after the primary injection, confirming the transient effect of the vaccination with TNFK. No toxicity related to TNFK active immunization was observed during the study period.

**Conclusion:** These data are the first demonstration of protection induced by TNFK in TTg mice on an established disease. We showed a reversibility of the TNF $\alpha$  inhibition by TNFK vaccination based on the observation of a spontaneous flare and the significant decrease of the anti-hTNF $\alpha$  neutralizing antibody titers over the study duration.

## 145 TWO STRUCTURALLY DIFFERENT PEPTIDES MIMIC A CD20 EPIOTOPE RECOGNIZED BY RITUXIMAB

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Besides its efficacy in the treatment of rheumatoid arthritis in double-blind controlled clinical trials, the anti-CD20 monoclonal antibody (mAb) Rituximab is emerging as an interesting therapeutic option for an increasing number of autoimmune diseases. However, antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity and apoptosis cannot exhaustively explain its therapeutic effects, as indicated by the lack of efficacy of other anti-CD20 mAb which recognize the same CD20 epitope as Rituximab. To provide a deeper insight of Rituximab function, we recently characterized two structurally different Rituximab-specific peptides: the 7-mer cyclic peptide Rp15-C bearing the Rituximab-specific antigenic motif <a/sNPS>, which matches the <sup>170</sup>ANPS<sup>173</sup> stretch of the exposed loop of CD20; and the 12-mer linear peptide Rp5-L, which express the Rituximab-specific antigenic motif <WPXWLE>. Here, we have addressed the issue as to whether the difference in the structure between Rp15-C and Rp5-L reflects the mimicry of the same or two different CD20-associated epitopes recognized by Rituximab. We show that, though Rp15-C and Rp5-L mimic the raft-associated form of CD20 and elicit anti-CD20 antibodies with a fluorescence pattern overlapping that of Rituximab, only anti-Rp5-L Abs recognize denatured CD20. Furthermore, the panning with anti-Rp5-L purified IgG with a phage display peptide library (PDPL), expressing cysteine constrained 7-mer peptide (c-7-c PDPL) and 12-mer linear peptide (12-mer PDPL), allowed the isolation of specific clones only with the second library. The results indicate that Rp5-L mimics a CD20-associated epitope different from that mimicked by Rp15-C and suggest that Rituximab recognizes two different CD20-associated epitopes. Implications of these findings on the therapeutic properties of Rituximab are discussed.

## 146 CALCIUM AND PHOSPHATE DELIVERY INTO OSTEOPOROTIC RAT TIBIA BY A NEW IONTOPHORETIC TREATMENT IN VIVO

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**Background:** Osteoporosis is one of the most common chronic disorders, which affects more than 750 million people all over the



world. In addition to pharmaceutical therapy and diet, physiotherapy may also be included in the management of osteoporosis.

**Objectives:** Our research group developed a novel iontophoretic instrument, as well as two chemically modified natural-based nanomolecules, as potential drugs for the local treatment of osteoporosis. The aim of our treatment was migrating  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  from the two donor nanomolecules into the osteoporotic bone by iontophoresis on rats in vivo. In our study we investigated the effects of the iontophoretic treatment with and without 17-beta-oestradiol therapy.

**Methods:** Thirty 3-month-old weanling Sprague-Dawley female rats, weighing approximately 200 g, were used in this study. Six rats were sham-operated (Group A) while the remaining rats were bilaterally ovariectomized under ketamine and xylazine anesthesia. After surgery the ovariectomized rats were divided into 4 groups: osteoporotic controls (Group B), animals treated by iontophoresis on the left tibia (Group C), rats treated by systemic 17-beta-oestradiol (s.c. 20 µg/kg) therapy (Group D), and animals treated by iontophoresis-oestradiol combination therapy (Group E);  $n = 6$  in each group.

For iontophoretic treatment a novel three-electrode iontophoresis instrument was used, which removes  $\text{Ca}^{2+}$  and  $\text{PO}_4^{3-}$  ions from the nanomolecules by their positive and negative charges. These ions were conducted through the skin and into the underlying bones using the third, reference electrode. In our studies 5 months after surgery we applied a total of five iontophoretic therapies on the left tibia of the rats, on every second day, for 30 minutes.

The effects of the treatment were investigated by ultrasound bone densitometry, biomechanical break tests, calcium and phosphate elemental analysis.

**Results:** Bone density, biomechanical parameters, the calcium and phosphate concentration of the osteoporotic controls (Group B) differed significantly from the results of the sham-operated rats (Group A) ( $p < 0.001$ ). Bone density of all of the treated groups (Group C, D, E) increased significantly compared to the osteoporotic controls ( $p < 0.05$ ). In Group C the maximum load, the stiffness and the stress biomechanical parameters significantly increased in the tibias treated by iontophoresis ( $p < 0.001$ ), just as in Group D ( $p < 0.05$ ) and Group E ( $p < 0.001$ ). The results of elemental analysis also showed differences in these groups versus the untreated Group B. There was no significant difference between the sham-operated Group A and the iontophoresis-oestradiol combination treated Group E.

**Conclusion:** Analyses revealed that the calcium and phosphate ions got into the treated bone by iontophoresis. The results of the biomechanical tests showed that the iontophoretic treatment increased the proof against fracture of the treated tibia. The new method in combination with oestradiol therapy was the most effective.

#### 147 IDENTIFICATION OF PREDICTIVE MARKERS OF RESPONSIVENESS TO INFlixIMAB AND ANAKINRA IN PATIENTS WITH ACTIVE RHEUMATOID ARTHRITIS

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**Background:** In the rheumatoid arthritis (RA) field, new therapeutic approaches, called immunotherapies, are now available (Infliximab, Anakinra, etc). These therapies affect the signalling pathways involved in RA: infliximab is a TNF $\alpha$  blocking agent whereas anakinra is an interleukin-1 receptor antagonist. However, the efficacy of a given drug in a given patient is variable and unpredictable. For these reasons, one of the major challenge of RA care is to predict the responsiveness to those drugs at an individual level.

**Objective:** To identify gene expression profiles able to predict the response to infliximab and to anakinra by using large scale analysis of gene expression from peripheral blood mononuclear cells (PBMC).

**Materials and methods:** Two independent sets of RA patients with active RA and resistant to conventional drugs were treated either by infliximab or by anakinra in association with methotrexate. The patients were categorized as responders whenever a 1.2 decrease of the Disease Activity Score (DAS 28) was observed at 3 months. Blood from responders and non-responders was collected at baseline before the onset of infliximab or anakinra treatment. Total RNAs were extracted from the PBMC, radiolabelled during reverse transcription, and hybridized over a set of 10,000 human cDNA array.

**Biostatistical analyses:** Hierarchical clustering, significance analysis of microarrays (SAM) and t-test were performed with the T-MEV software. Finally, selected transcripts were re-assessed by qRT-PCR in an independent set of patients.

**Results:** In 6 responders and 7 non-responders to infliximab, 41 mRNAs identified were expressed as a function of the response to treatment. The expression levels of 20 of them (measured by qRT-PCR) were able to accurately classify 16 out of 20 other patients (10 responders and 10 non-responders), with a specificity of 70% and a predictive negative value of 90%. In 7 responders and 7 non-responders to anakinra, 52 mRNAs identified were expressed as a function of the response to the treatment. The expression levels of 20 of them (measured by qRT-PCR), were able to accurately classify 14 out of 18 other patients (9 responders and 9 non-responders), with a specificity and a predictive negative value of 80%.

**Conclusion:** Here, we identified and validated for the first time two combinations of genes able to predict responsiveness to infliximab (Lequerré T *et al. Arthritis Res Ther* 2006; Patent n°EP 06290789.4) and anakinra. Identification of markers of responsiveness to other immunotherapies is currently ongoing. The predictive gene profiles for each molecule could be deposited on a same support (mini-transcriptome) to test the response of a given patient to these drugs with only a blood sample. This approach opens new ways for RA patients; it will allow optimizing RA care by prescribing these treatments only to expected responder patients, avoiding severe side effects to non responder patients and finally limiting wasteful expenditures.

*\*Both authors contributed equally to the work.*

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#### 148 PLASMID DNA-CYTOFECTIN COMPLEXES ENCODING THE STRESS PROTEIN BiP AS A POTENTIAL THERAPY FOR RHEUMATOID ARTHRITIS

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Rheumatoid arthritis (RA) is a chronic autoimmune disease manifesting primarily as an inflammatory, polyarthritis of the synovial joints. While biologic therapies such as anti-TNF $\alpha$  have revolutionised the treatment of RA, such therapy is not effective in all patients and there is an urgent need to investigate novel therapies for RA. BiP is a 78 kDa endoplasmic reticulum (ER) stress protein which demonstrates immunomodulatory activity. When administered to mice with collagen induced arthritis (CIA), recombinant human BiP protein can both prevent the onset of, and treat ongoing arthritis. BiP mediates these effects through the induction of a broad profile of anti-inflammatory genes, including IL-10.

As a novel, non-invasive gene therapy for RA we are investigating nasal administration of plasmid DNA-cytofectin complexes encoding murine (m) BiP, based on the hypothesis that transfection of myeloid lineage cells within the nasal associated lymphoid tissue has the potential to deliver genes to sites of inflammation. To this

end, 9 silent mutations were introduced into the mBiP gene to discriminate between vector derived and endogenous BiP transcripts and the C-terminus KDEL sequence has been removed from the gene, permitting protein secretion. The mutant mBiP gene has been cloned into the pGWIZ and pIRES:eGFP expression vectors and protein expression has been validated by transfection of 293T cells *in vitro*. In murine ATDC5 cells, vector derived and endogenous BiP transcripts are discernable by RT-PCR. Biological activity of mBiP protein has been demonstrated by applying supernatants and lysates from pGWIZ or pgBiP transfected cells to human PBMC. PBMC respond to murine BiP by secreting the anti-inflammatory cytokine IL-10. Further studies will investigate the effects of vector administration in naïve DBA/1 mice and ultimately in the collagen induced arthritis model of RA.

#### 149 TRANSDUCTION OF HUMAN FIBROBLAST-LIKE SYNOVIOCYTES WITH ADENO-ASSOCIATED VIRUS SEROTYPE 5 IS PERSISTENTLY INCREASED AFTER PROTEASOME INHIBITION: A POSSIBLE APPLICATION FOR RHEUMATOID ARTHRITIS *IN VIVO* GENE THERAPY

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**Background:** Adeno-associated virus (AAV) is currently considered as one of the most potent vectors for human gene therapy. We are developing local gene therapy for the treatment of rheumatoid arthritis (RA). We have shown that recombinant AAV serotype 5 (rAAV5) is the most efficient vector in transducing fibroblast-like synoviocytes (FLS), the most resident cells in the joint. In addition, we showed that rAAV5 expressing a TNF-blocking agent ameliorates disease activity in animal models of arthritis. Previously, it has been described that proteasome inhibition transiently enhances single-stranded (ss) rAAV2-mediated transgene expression in RA FLS. We investigated if proteasome inhibition also affects ss and self-complementary (sc) AAV5-mediated transgene expression in primary human RA FLS. We also investigated the influence of synovial fluid (SF) hereon.

**Methods:** RA FLS were transduced with  $25 \times 10^5$  vp/cell of ssAAV5.GFP, scAAV5.GFP and ssAAV5.TNFRI (UNC vector core, North Carolina). After 36 hours, the proteasome inhibitor MG132 (40  $\mu$ M) was added, with or without SF (12.5%), for 16 hours. GFP expression was analyzed up to day 21 after transduction by a semi-quantitative scoring system (0 = 0–10%, 1 = 10–30%, 2 = 30–60%, 3 = 60–80%, 4 = 80–100% expression) using a fluorescent microscope and by FACS-analysis. TNFRI secretion in the culture medium was measured by ELISA.

**Results:** Without any addition almost no transgene expression was observed using both the ss and sc rAAV5.GFP vector. MG132 enhanced GFP expression already 3 days after transduction and this was increased at day 9 both with ssAAV5.GFP ( $0.8 \pm 0.4$ ,  $n = 5$ ) and scAAV5.GFP ( $2 \pm 0.7$ ,  $n = 5$ ), and sustained until at least day 14. Comparable expression patterns were observed after FACS analysis of the cells at day 9. SF alone did not have any effect on the transgene expression. Using ssAAV5.TNFRI, the protein secretion was significantly increased in the culture media 3 and 7 days after transduction in the presence of MG132.

**Conclusion:** We have demonstrated that modulation of the proteasome system in primary human RA FLS increases gene expression of rAAV5 and that this increase in expression is persistent. In addition, we show for the first time that this effect is even more pronounced using a scAAV5. Modulation of the proteasome pathway and the use of sc vectors may also be beneficial in *in vivo* rAAV5-mediated gene delivery to synovial tissue of RA patients.

#### 150 PSYCHOLOGICAL ASSESSMENT OF PATIENTS WITH SCLERODERMA

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**Introduction:** Scleroderma is a chronic and rare disease and psychological factors such as anxiety, depression, guilty feelings and social functionality may have great consequence. Research on emotional distress in systemic sclerosis showed that depressive and anxiety feelings are not only related to objective measures of illness such as lung problems, esophageal involvement, total skin score, gastrointestinal index, but to personality traits as well (Roca *et al*, 1996; Angelopoulos *et al*, 2001; Matsuura *et al*, 2003; Richards *et al*, 2004; Nietert *et al*, 2005; Hyphantis *et al*, 2007).

**Aim of the study:** The aim of the study was to identify emotional disorders, personality characteristics, parameters of social support, ways of coping and their interactions, in patients with systemic scleroderma.

**Materials and methods:** Sixty two patients participated in the study, and were recruited from the rheumatologic outpatient clinic of Laiko University Hospital of Athens. They were all classified as SSc patients based on the American College of Rheumatology criteria. Healthy controls ( $n = 74$ ) were recruited from working staff and their relatives of a private clinic in Athens and were matched in age and sex with patients. Demographic data were collected from both groups. The instruments that were used in the research were: Center of Epidemiological Studies of Depression (CES-D), Hospital Anxiety And Depression Scale (HADS), Eysenck Personality Questionnaire (EPQ), Short form of Social Support (SSQ), Life Experiences Survey (LES), Ways of Coping (WOC).

**Results:** Patients scored higher on depression and anxiety than controls, whereas they scored less on a dimension of personality, extraversion. Patients and controls differed significantly on social support satisfaction, on negative and positive life events mentioned, as well as on the scores of those events. Differences were also displayed on ways of coping styles, with patients using more wishing and seeking of divine, and less assertiveness, problem solving and positive reappraisal.

**Conclusions:** Patients with scleroderma are more prone to depression and anxiety feelings. In this sample, they were found to be less extrovert than healthy controls. Patients with scleroderma experience more frequently negative events in the preceding year that are more intense than those experienced by healthy controls. Patients experience more rarely positive events and with less intensity than those experienced by healthy controls. Patients demonstrate weaker ways of coping in stressful events in relation to healthy controls.