1. Immunological tolerance and autoimmunity

A1 ALTERED PHAGOCYTIC ACTIVITY IN PRIMARY SJÖGREN’S SYNDROME: SIMILARITY TO LUPUS AND ASSOCIATION WITH EXTRAGLANDULAR DISEASE

GE Frangoulis, M Tami, M Herrmann, HM Moutsopoulos, MN Manoussakis.
Department of Pathophysiology, National and Kapodistrian University of Athens, Athens, Greece; Institute for Clinical Immunology, University of Erlangen, Nuremberg, Germany

Background and Objectives: The pathogenesis of systemic lupus erythematosus (SLE) has been thought to involve impaired clearance of apoptotic remnants, as suggested by altered uptake capacity of peripheral blood monocytes and granulocytes. In this study, we sought to assess the phagocytic capacity of peripheral blood monocytes and granulocytes of patients with primary Sjögren’s syndrome (SS). For this purpose, we examined comparatively the uptake of fluorescent microbeads and of necrotic cell-derived material in SS patients, SLE patients and healthy controls.

Methods: Patients studied included 29 with primary SS (American–European criteria 2002) and 13 with SLE (American College of Rheumatology criteria 1997). Healthy blood donors (HBD) matched for age and sex to the SS and SLE groups (HBD-1, n = 15 and HBD-2, n = 13, respectively) were also studied in all assays. The phagocytosis index was calculated according to previously established methods (Gaal et al, J Autoimmun 2007) using heparinised whole blood from individuals studied mixed with a commercially available preparation of fluorescent microbeads and of necrotic cell-derived material (MB-phagocytosis; Polysciences, size 1 μm, 25 SS and 13 SLE patients studied) or a preparation of propidium iodide-stained necrotic cell-derived material obtained from heat-treated normal peripheral blood mononuclear cells (SNEC-phagocytosis, 11 SS and 8 SLE patients studied). The ingestion of particles was assessed by flow cytometry and the phagocytosis index (Plnd) was calculated as the product of the percentage of fluorescent cells by the mean fluorescence intensity.

Results: In agreement with previous studies, SS patients manifested significantly decreased capacity for MB-phagocytosis by granulocytes (median Plnd 18, range 5–47), as well as by monocytes (median Plnd 30, range 1–98), compared with HBD-2 (granulocytes: median Plnd 53, range 42–110, p = 0.002; monocytes: median Plnd 108, range 90–182, p = 0.0001). In SS patients, defective MB-phagocytosis involved only monocytes (median Plnd 26, range 1–126), compared with HBD-1 (median Plnd 113, range 70–191, p<0.0001). On the other hand, significantly increased SNEC-phagocytosis was observed in granulocytes (but not monocytes) in SS (median Plnd 419, range 270–1018), as well as in SLE (median Plnd 415, range 155–1487), compared with HBD-2 (median Plnd 153, range 76–244, p<0.0001) and HBD-1 (median Plnd 154, range 86–287, p = 0.001), respectively. MB-phagocytosis by monocytes was significantly decreased in SS patients with extraglandular manifestations (median Plnd 23, range 1–58) compared with SS patients without (median Plnd 39, range 10–126, p = 0.02).

Conclusions: The peripheral blood phagocytes of SS patients appear to manifest altered capacity for the uptake of microbeads and SNEC, in a manner similar to SLE. Such disordered phagocytosis may play a role in the pathogenesis of SS.

A2 ANTIGEN-SPECIFIC T CELLS TRANSFER PRISTANE-INDUCED ARTHRITIS TO NAIVE RECIPIENTS

MH Hoffmann, B Kusenda, C Baumann, G Steiner. 1Division of Rheumatology, Internal Medicine III, Medical University of Vienna, Vienna, Austria; 2Max F Perutz Laboratories, Vienna, Austria; 3Center of Molecular Medicine, Austrian Academy of Sciences, Vienna, Austria

Background and Objectives: Heterogeneous nuclear ribonucleoprotein-A2 (hnRNPA2) is known to be a B and T-cell autoantigen in rheumatoid arthritis (RA) and was recently demonstrated to be among the primary targets of autoimmunity in pristane-induced arthritis (PIA) in the DA.1F rat (Hoffmann et al, J Immunol 2007;179:7568–76). Autoantibodies to hnRNPA2 as well as autoreactive T cells showing a Th1-like phenotype are present in pristane-injected rats already one week before disease onset. Remarkably, hnRNPA2 was also shown to stimulate non-pristane-primed lymph node cells to produce inflammatory cytokines in a MyD88-dependent manner suggesting the involvement of Toll like receptors (TLR). The aim of this study was to gain more information about the functional and pathogenic relevance of autoimmunity against hnRNPA2 in rats with PIA.

Materials and Methods: Splenocytes and cells from inguinal lymph nodes of DA.1F rats were extracted 14 days after the intradermal injection of pristane, restimulated with hnRNPA2, the closely related proteins hnRNPA1 and hnRNPA3 and control antigens (vimentin and BSA) in vitro, and transferred to irradiated recipient rats. Body weight and clinical arthritis were scored twice a week over a period of 50 days after cell transfer. Joint sections were stained with haematoxylin and eosin, toluidine blue, for activity of tartrate-resistant acid phosphatase and with an hnRNPA2-specific monoclonal antibody. A TLR ligand screening was performed using cell lines expressing only one TLR along with a recombinant reporter gene.

Results: Lymphocytes isolated from pristane-primed rats and restimulated in vitro with hnRNPA2, hnRNPA1, or hnRNPA3 induced arthritis in naive DA.1F rats, whereas control antigens were ineffective. The disease started 5–9 days after intravenous cell transfer and clinically and histologically closely resembled PIA. The data obtained from the TLR ligand screening suggested that TLR7 might be specifically involved in the autoimmune response to hnRNPA2. As TLR7 is a RNA-binding receptor we assume that the RNA component of hnRNPA2 binds and stimulates TLR7, whereas the protein is (aberrantly) presented by dendritic cells to autoreactive T cells, which subsequently induce and/or enhance the inflammatory cascade in PIA. Studies are underway to identify the RNA bound by hnRNPA2 under normal and pathological conditions.

Conclusion: Taken together, the early presence of humoral and cellular autoimmune responses to hnRNPA2, its abundant expression in the inflamed joint, its immune stimulatory capacity and, most importantly, the capability of hnRNPA2-stimulated lymphocytes to transfer arthritis passively suggest that hnRNPA2 plays a major role in the pathogenesis of arthritic disorders such as PIA and RA. The new data thus provide evidence for a functional involvement of hnRNPA2 and related proteins in the pathogenesis of destructive arthritis.

A3 CELLS FROM A VANISHED TWIN AS A POSSIBLE SOURCE OF MICROCHIMERISM IN MEN WITH SCLERODERMA?

LM de Belffion, P Heiman, D Azzou, J Jak, M Martin, J Roudier, F Roufousse, NC Lambert. 1Institute for Medical Immunology, Université libre de Bruxelles, Brussels, Belgium; 2Laboratory of Cytogenetics, Hôpital Erasme, Université libre de Bruxelles, Brussels, Belgium; 3INSERM Unit 639, Marseille, France; 4Hôpital de la Conception, Service de Rheumatologie, Marseille, France; 5Département de Médecine Interne, Hôpital Erasme, Université libre de Bruxelles, Brussels, Belgium

Background and Objectives: Microchimerism is the presence in small quantities of cells and/or DNA genetically different from that of the host. Natural microchimerism occurs during pregnancy; fetal cells cross the placenta barrier to reach the maternal circulation and persist decades after delivery as fetal microchimerism. In contrast,
maternal cells persist in adults as the imprint of in-utero life. Gravid women therefore potentially host at least two sources of microchimerism. A decade ago it was suggested that microchimerism could sometimes be detrimental to the host and explains female predominance in autoimmune diseases such as systemic sclerosis (SSc). SSc is indeed very rare in men. We propose to test the hypothesis that in addition to maternal microchimerism, men with SSc may have another source of microchimerism from a vanished twin, which might be a risk factor for SSc.

Methods: The presence of maternal or twin cells is tested by fluorescence in situ hybridisation (FISH) and the presence of foreign DNA is quantified by real time HLA-specific PCR for the non-inherited maternal or paternal HLA antigens (NIMA or NIPA) in peripheral blood mononuclear cells (PBMC) from men with SSc and healthy men.

Results: In preliminary studies, we have shown by FISH the presence of female cells in PBMC from a man with SSc. Furthermore, we proved by HLA-PCR the presence of twin cells on the basis of the presence of microchimerism for NIPA, but not maternal microchimerism in that patient.

Conclusions: Cells from a vanished twin might be a possible source of microchimerism in men with SSc. We wonder if this phenomenon is anecdotal or corresponds to a common pattern in men with SSc. Further studies on other patients and isolation of these chimeric cells will give a full answer.

Background and Objectives: We have shown earlier that a human cartilage aggrecan peptide (P135) containing the shared epitope was arthritogenic in BALB/c mice, and it was also recognised in HLA-DR4 humanised mice. In the present study we investigated the presence and significance of differential T-cell recognition of HLA and cartilage aggrecan peptides containing non-citrullinated or citrullinated variants of the shared epitope sequence.

Materials and Methods: ELISPOT assay was used to detect IL-17 response to citrullinated and non-citrullinated synthetic peptides representing the shared epitope-containing HLA DRB1 alleles and P135 as well as shared epitope-free aggrecan sequences. Flow cytometric bead-based multiplexed assays were used to measure soluble Th1 and Th2 cytokines in tissue culture supernatants of unstimulated and peptide stimulated cells of patients with rheumatoid arthritis (RA) and healthy controls.

Results: In vitro stimulation of peripheral blood mononuclear cells with shared epitope-free aggrecan peptides resulted in a significantly reduced IL-17 response both in patients and in controls. Citrullinated or non-citrullinated peptides representing the shared epitope of HLA DRB1 did not have a significant effect on IL-17 production. In contrast, the P135 aggrecan peptide induced a significant IL-17 response in patients with RA (p<0.001). Furthermore, in some controls (n = 5/15) and patients with RA (n = 6/15) citrullination of P135 led to a three to fourfold increase in the baseline IL-17 response. Citrullination also significantly influenced aggrecan and HLA peptide recognition by sera of RA patients and controls.

Conclusions: In addition to the plethora of data on citrullination-related antibodies in RA, our study provides evidence for differential recognition of citrullinated altered peptide ligands by T cells.

Background: Lupus arthropathy is characterised by a chronic non-erosive non-deforming (NEND) arthropathy or a deforming non-erosive arthropathy, whereas the erosive type represents an overlap disease with rheumatoid arthritis, called rhupus.

Aim: To define clinical and immunological features of each one of the different forms of arthritides occurring in systemic lupus erythematosus (SLE).

Methods: 521 patients with SLE were retrospectively evaluated. The occurrence of anti-extractable nuclear antigen antibodies, C-reactive protein (CRP), erythrocyte sedimentation rate, rheumatoid factor and anti-cyclic citrullinated peptide (CCP) were assessed. Clinical and laboratory data were derived from medical records.

Results: 298 out of 521 patients with SLE showed chronic arthritis (57.2%): 253 of them NEND arthritis (84.9%), 20 mild deforming non-erosive arthritis (6.7%), 15 deforming (Jaccoud) arthropathy (JA) (5%) and 10 erosive arthritis (5.3%) (rhupus). No differences in sex distribution, age at onset or extra-articular involvement were recorded between the different groups of arthritides, except for Raynaud’s phenomenon more rarely reported in the NEND arthritis group compared with rhupus and mild deforming arthropathy (p = 0.006 and p = 0.005, respectively). Patients with JA and with mild deforming arthropathy showed a longer disease duration (p = 0.0000003 and p = 0.008, respectively) compared with the NEND arthritis group. Rheumatoid factor was more frequently detected in rhupus (60%) than in the JA group (35%) (p = 0.0068) or in NEND arthritis patients (17%) (p = 0.0026). By contrast, anti-CCP antibodies did not show a significantly different distribution between groups, even if they were more frequently detected in rhupus patients. Nevertheless, the titre of anti-CCP antibodies in rhupus cases was lower compared with the titre of the other groups (p = 0.03). CRP elevation was more frequently recorded in JA (73.3%) compared with mild deforming arthropathy (30%) (p = 0.023) and NEND arthritis (31.2%) (p = 0.0021). On the contrary, anti-Ro/57gene syndrome A antibodies and lupus anticoagulant are not associated either with JA or mild deforming arthropathy.

Conclusion: JA is an inflammatory manifestation of SLE, probably consequent to the CRP activation pathway, different from other inflammatory lupus features, such as renal or cutaneous involvement. The frequent occurrence of rheumatoid factor in the rhupus group confirms the overlap with RA. Nevertheless, the classification of patients with lupus arthropathy is not easy, because many features are shared by different types of arthritis (such as radiological features of RA and rhupus).

Background and Objectives: Our body is equipped with a natural protective mechanism that prevents unwanted immune reactions, which is mediated by special immune cells, regulatory T cells (Treg). An imbalance in the action of Treg could, therefore, leads to autoimmune disease, such as rheumatoid arthritis (RA). Indeed, it has been shown that the action of Treg is affected in RA patients. We have shown, in animal experiments, that the infusion of Treg can be an efficient treatment for arthritis. A problem, however, for the application of such a treatment is the difficulty of growing Treg
in sufficient numbers, and, in cases derived from RA patients, their hampered function. Recently, it has been shown that murine T cells can be converted into FOXP3+ Treg by all-trans retinoid acid (ATRA). The goal of this study was to develop an efficient approach to generate large numbers of CD4 Treg with potent and stable suppressive ability from adult human peripheral blood mononuclear cells (PBMC).

Materials and Methods: To investigate the effect of ATRA on the de novo generation of Treg as well as on the function of expanded CD4+CD25++ Treg isolated from PBMC, we purified total CD4+CD25−, CD4+CD25−CD45RA+− naive/memory effector cells as well as CD4+CD25++ Treg from adult PBMC by FACS-sorting. Subsequently, these cells were stimulated in the absence/presence of transforming growth factor beta (TGFβ) and/or ATRA. After restering for differentiation, the phenotype and function of these cells were determined by FACS staining and standard in vitro suppression assay, respectively.

Results: Although the addition of ATRA had no effect on the initial proliferation of peripheral CD4 T cells, these cells displayed significantly reduced proliferative ability upon re-stimulation. Moreover, in the presence of exogenous TGFβ, ATRA consistently generates CD4 Treg from naive CD4+CD25−CD45RA+− conventional cells isolated from PBMC. These ATRA-induced Treg displayed high and stable levels of FOXP3 expression, and more importantly, potent and stable suppressive capacity as they inhibited the proliferation of autologous effector cells to a comparable extent as did ex vivo isolated CD4+CD25++ Treg. Furthermore, the addition of ATRA and TGFβ during the stimulation of purified CD4+CD25++ Treg significantly preserves/enhances their suppressive capacity in long-term culture.

Conclusions: Our data show that ATRA not only efficiently induces the de novo generation of Treg from naive human effector T cells, but also enhances the suppressive function of isolated CD4+CD25++ naturally occurring Treg. These indicate that stable and potent Treg can be obtained in RA patients by ATRA, which is relevant as it has been described that the function of Treg in RA patients is compromised.

A7 Detection of anti-serum amyloid autoantibodies on Western Blots implies a potential physiological role

K Lakota, KM Poljšak, T Kveder, B Rozman, SS Šemrl. University Medical Centre, Division of Internal Medicine, Department of Rheumatology, Ljubljana, Slovenia

Background: Serum amyloid A (SAA) is an acute phase protein, markedly increased during acute and chronic inflammation in animals and humans. SAA has been indicated to represent a prediction marker for subacute stent thrombosis and a prognostic marker for increased mortality in acute myocardial infarction patients. Certain anti-acute phase protein autoantibodies (anti-C-reactive protein antibodies in systemic lupus erythematosus (SLE)) have been associated with physiological roles of clearance. In 2004, a report indicated the occurrence of anti-SAA autoantibodies (anti-SAA) by ELISA and their association with a variety of cardiovascular conditions, among them deep vein thrombosis, but also SLE. However, since then, there has been a general lack of information concerning the presence of anti-SAA in either healthy donors and/or patients.

Aims of Study: To determine the presence/absence of anti-SAA autoantibodies by immunoblotting in sera of healthy donors, patients with autoimmune diseases and patients with venous and/or arterial thrombosis and to compare the results.

Materials and Methods: A total of 156 human sera was tested. There were 69 blood donors, and patient groups (total n = 87) with set diagnoses were divided into two groups (primary antiphospholipid syndrome, secondary antiphospholipid syndrome, SLE and rheumatoid arthritis were joined into the autoimmune group; venous thrombosis and arterial thrombosis were joined into the thrombosis group of patients) and compared for the presence of anti-SAA. Human recombinant SAA was loaded onto 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane and cut into strips. Blood donor and patient sera were incubated at a dilution of 1 : 50, followed by secondary goat anti-human IgG-alkaline phosphatase at a dilution of 1 : 1000. Detection of anti-SAA was performed with NBT/BCIP and colorimetry. As a positive control anti-human SAA conjugate with alkaline phosphatase was used. The specificity controls were performed.

Results: Surprisingly, 95% of thrombotic patients and 81% of all blood donors tested by immunoblot analysis were anti-SAA positive. The highest percentage of negatives (23%) was found in the autoimmune disease group of patients.

Conclusions: In the future, the number of patients within the groups would need to be expanded further and immunoblotting data supplemented with ELISA, which could lead to the elucidation of potential physiological roles of anti-SAA.

A8 Expanded proinflammatory T cells in inclusion body myositis

J Pandya, A Fasth, S Amardottir, E Lindroos, E Lundberg, V Malmström. Rheumatology Unit, Department of Medicine, Karolinska University Hospital, Solna, Sweden; Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden

Inclusion body myositis (IBM) is a chronic, inflammatory myopathy of unknown cause, which is characterised clinically by muscle weakness and muscle atrophy, particularly of the quadriceps and finger flexor muscles, and by being resistant to conventional immunosuppressive drugs. The muscle tissue of IBM patients is characterised histopathologically by leucocyte infiltrates, preferentially T cells and macrophages. We have previously demonstrated the accumulation of a specific phenotype of T cells, so-called CD28null T cells, in muscles of patients with dermatomyositis and polymyositis. The CD28null T cells are apoptosis resistant, pro-inflammatory and cytolytic cells that hypothetically may also play a role in the disease mechanisms of IBM. We thus investigated the frequency and effector functions of CD28null T cells in IBM and whether this subset of T cells was clonally expanded.

Patients and Methods: A cohort of 20 patients with IBM was analysed for the frequency of circulating CD4+CD28null and CD8+CD28null T cells in peripheral blood and muscle biopsies taken at different time points during the disease course. The TCR-Vβ usage was determined by the flow cytometry-based IOTest1 B Mark kit (n = 6). For functional analysis, peripheral blood mononuclear cells (PBMC) were polyclonally stimulated with plate-bound anti-CD3 for 6 h and 72 h (n = 5) and the frequencies of intracellular IFNγ and CD107a (a marker of degranulation and cytotoxicity) containing T cells were recorded by multicolour flow cytometry.

Results: CD28null T-cell populations were clearly expanded in peripheral blood of IBM patients and both the CD4 and CD8 CD28null T-cell populations were highly TCR-Vβ restricted compared with the CD28+ subsets from the same patient. Different patients displayed different TCR-Vβ restrictions and the expansions were consistent over time. Anti-CD3 stimulation of PBMC resulted in a fast and high frequency of CD28null T cells of both the CD4 and CD8 subsets significantly more positive for IFNγ and CD107a compared with CD28+ subsets. Both the TCR-Vβ-dominant and non-dominant CD28null subsets were equally activated under these conditions.

Conclusions: The TCR-Vβ restriction of CD28null T cells in IBM patients suggests that a limited number of antigens is involved in driving the expansion and high TCR-Vβ restriction of CD28null T cells in IBM patients. Functional evaluation experiments revealed...
that CD28null T cells in both the CD4 and CD8 compartment are devoted proinflammatory and cytotoxic effector cells. Interestingly, the TCR-Vβ-dominant and non-dominant CD28null T-cell subsets displayed an equal quality and quantity of effector function. Further analysis is ongoing to evaluate the antigen specificity of these CD28null T cells and their presence in skeletal muscle infiltrate of IBM patients. Functional characterisation of proinflammatory T cells will provide important clues for designing new T-cell-targeted therapies for inflammatory myopathies.

**A9 HYDROGEN BONDS ARE CRUCIAL FOR THE ANTI-BETA 2-GLYCOPROTEIN I ANTIBODY BINDINGS TO THE ANTIGEN**

1Jurgec, 2M Lunder, 4M Bratkovič, 5 S Čuñik, 7 T Kveder, 8B Rozman, 1,8B Božič.
1University Medical Centre, Department of Rheumatology, Ljubljana, Slovenia; 2University of Ljubljana, Faculty of Pharmacy, Ljubljana, Slovenia

**Background and Objectives:** The interactions between β2-glycoprotein I (β2-GPI) and anti-β2-GPI antibodies had been widely studied, but still remain unclear. The aim of our study was to determine the nature of high avidity anti-β2-GPI paratopes by the phage display method.

**Materials and Methods:** Purified high avidity anti-β2-GPI IgG derived from plasma obtained by plasmapheresis of an antiphospholipid syndrome patient and β2-GPI were used to screen a cyclic heptamer phage display library (New England Biolabs, Ipswich, MA, USA). Single-stranded DNA from amplified selected phage clones were isolated and sequenced (MWG Biotech, Munich, Germany).

**Results:** Selection of anti-β2-GPI or β2-GPI binding peptide sequences by phage display yielded a series of sequences with high content (more than 60%) of polar amino acids, especially those with an hydroxyl functional group on a side chain (T, S, D, rarely Y). The most frequently observed motif for β2-GPI binding peptide sequences was Thr/ Ser–Xaa–Xaa–A/Ala (T/S)–X–X–(A/P), where X represents any amino acid, where hydroxyl containing (T, S, D) or basic (H, R, rarely K) was the most common. The characteristic for high avidity anti-β2-GPI was a conserved motif Thr–Thr/ Ser–Xaa–Val/Pro (T–(T/S)–X–(V/P).

**Conclusions:** Using a cyclic heptamer peptide phage display library, similar motifs on high avidity anti-β2-GPI and β2-GPI could be identified. Motif T–(T/S)–X–(V/P) represents an antigenic structure recognised by anti-β2-GPI, whereas (T/S)–X–X–(A/P) represents putative amino acids in paratopes of anti-β2-GPI interacting with β2-GPI epitopes. We suggest that the formation of hydrogen bonds between amino acids, which have basic characters or have an hydroxyl functional group on the side chain is crucial for the interaction between high avidity anti-β2GPI and β2-GPI.

**A10 ORAL DELIVERY OF SPECIFIC ALPHA 7 ACETYLCHOLINE RECEPTOR AGONISTS THAT DO NOT PASS THE BLOOD–BRAIN BARRIER AMELIORATES COLLAGEN-INDUCED ARTHRITIS IN MICE**

1M van Manen, 2J Koepe, 3L Bevaert, 4G LaRosa, 5M Vervoordeldonk, 6P Tak. 1Division of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; 2Arthrogen BV, Amsterdam, The Netherlands

**Objective:** The cholinergic nervous system may downregulate inflammation in vivo by decreasing the release of proinflammatory cytokines by activated macrophages. It has been suggested that the vagus nerve might exert its anti-inflammatory effects by a specific effect of its principal neurotransmitter acetylcholine on the α7 subunit of nicotinic acetylcholine receptors (nAchR) on macrophages and other immune cells. We have previously shown in murine collagen-induced arthritis (CIA) that stimulation of the α7nAchR by intraperitoneally injected AR-R17779 can reduce the severity of arthritis, indicating that the α7 subunit plays an important role in synovial inflammation. The objective of this study was to investigate further the role of the cholinergic nervous system in CIA using two α7-specific agonists, CTI-15511 and CTI-15072. The compound 15511 is more potent (2 nmol) in binding to alpha 7 than 15072 (8 nmol). Furthermore, CTI-15511 shows ion channel agonist activity, which is not seen with CTI-15072, and both compounds hardly pass the blood–brain barrier.

**Methods:** CIA was induced in DBA/1 mice at day 0 by immunisation with bovine collagen type II (bCII) followed by a booster injection with bCII on day 20. CTI-15511 (2, 5 and 10 mg/kg) and CTI-15072 (5, 10 and 20 mg/kg) were administered daily with oral gavage from day 20 till mice were killed on day 54 (n = 15 per dose). Control mice (n = 15) received saline. Disease progression was monitored by visual clinical scoring and measurement of paw swelling. In addition, the effects on bone degradation, histological joint damage and inflammation were assessed.

**Results:** In mice treated with 2 mg/kg CTI-15511 a 54% reduction in clinical arthritis and a significant delay in the onset of disease was observed (p<0.05). Moreover, paw swelling was significantly decreased (p<0.05). Treatment with higher doses of CTI-15511 showed comparable effects, albeit less pronounced. Radiographic analysis showed significantly less joint destruction in the knee joints (p<0.005). Synovial tissue inflammation was also significantly decreased (p<0.05). For CTI-15072 the most potent effect was observed at 5 mg/kg showing a reduction of 36% in the arthritis score. Higher doses did not show a significant effect.

**Conclusions:** These data show that CTI-15511 can reduce the severity of arthritis, indicating that the α7 subunit plays an important role in synovial inflammation. As CTI-15072 is less effective this indicates that alpha 7 ion channel activity may be of importance in driving the anti-inflammatory response. Because the effect of CTI-15311 on the central nervous system is negligible, the therapeutic effect is most likely based on binding of CTI-15311 to peripheral α7 subunits of the nAchR, which may be a future target for the treatment of rheumatoid arthritis.
PLASMACYTOID DENDRITIC CELLS INDUCE POTENT REGULATORY T CELLS FOLLOWING SUCCESSFUL THERAPY IN RHEUMATOID ARTHRITIS PATIENTS

M Kavousanaki, I Kriklos, E Choustoulaki, P Sidiropoulos, D Bourpas, P Verginis. Laboratory of Autoimmunity and Inflammation, Rheumatology, Clinical Immunology and Allergy, University of Crete, Crete, Greece

Objectives: Recent data have shown that regulatory T cells (Treg) from patients with active rheumatoid arthritis (RA) are decreased in numbers or functionally defective, but their levels and function are restored in patients responding to therapy. Although dendritic cells (DC) have been implicated in the maintenance of self-tolerance, the precise mechanisms underlying this phenomenon are poorly defined. Therefore, the aim of this study was to elucidate the participation of DC in the induction and expansion of Treg in RA patients.

Methods: The phenotype of isolated DC subsets from the peripheral blood of RA patients (n = 25) and healthy individuals (n = 10) was assessed by flow cytometry using markers specifically expressed on myeloid or plasmacytoid DC. Subsequently, plasmacytoid and myeloid DC from inactive RA patients and healthy controls were cultured with allogeneic naive CFSE-labelled T cells, to examine the ability of DC to polarise naive T cells. The readout of these assays was the proliferation of T cells based on CFSE dilution and their cytokine profile, by sandwich ELISA. Expression of the immunoregulatory indoleamine 2,3-dioxygenase (IDO) enzyme by DC was assessed with quantitative reverse transcriptase PCR and its effect on T-cell polarisation was examined by using an IDO antagonist. Disease activity was assessed by the disease activity score (DAS28). Remission was defined as a score of 2.4 or less.

Results: In active RA patients, CD303+ plasmacytoid DC and CD1c+ myeloid DC cell populations are undetectable in the periphery (0.07% and 0.25%, respectively) but they re-establish in patients who have achieved remission (0.54% and 5.94%). Mature plasmacytoid DC isolated from inactive patients induced a limited proliferation of allogeneic naive CD4 T cells and elevated the expression of IDO enzyme in vitro, whereas myeloid DC induced a robust proliferation of T cells. Furthermore, T cells polarised in the presence of plasmacytoid DC but not myeloid DC secreted high levels of IL-10 in an IDO-dependent fashion, because the neutralisation of IDO activity in vitro abrogated this phenomenon. Collectively, these data provide evidence that plasmacytoid DC, from RA patients who have undergone remission, polarise naive T cells towards a Treg phenotype.

Significance: Our findings demonstrate, for the first time, an intrinsic ability of plasmacytoid DC to regulate T-cell responses during autoimmunity in humans and support the use of plasmacytoid DC as a potential therapeutic modality for autoimmune diseases and transplantation.

PREVALENCE OF AUTOANTIBODIES AND AUTOIMMUNE DISEASES IN PATIENTS WITH HEPATITIS C VIRUS INFECTION TREATED WITH INTERFERON ALPHA

M Smyrka-Kaczmarek, M Klimczak, I Dziemianko, K Simon, I J Szechinski. 1Department of Rheumatology and Internal Diseases, Wroclaw Medical University, Wroclaw, Poland; 2Department of Hepatology and Infectious Diseases, Wroclaw University of Medicine, Wroclaw, Poland; 3Department of Nephrology and Transplantation Medicine, Wroclaw University of Medicine, Wroclaw, Poland

Background and Aims: Hepatitis C virus (HCV) infection is associated with various autoimmune phenomena more often than other viral diseases. It is characterised by the synthesis of many organ-specific and non-organ-specific autoantibodies, which is often clinically silent but sometimes accompanied by overt autoimmune disease. They include mixed cryoglobulinaemia, lymphocytic syladenitis resembling Sjögren syndrome, polyarthritis, thyroid diseases, autoimmune cytopenias and others. The therapy of hepatitis C with interferon α may also induce autoimmune complications in susceptible patients. The side effects of this treatment described so far include the induction of autoantibodies and development of autoimmune thyroïditis, diabetes mellitus, myasthenia gravis, coeliac disease, systemic lupus erythematous, arthritis, immune-mediated skin diseases and others. The aim was to evaluate the prevalence of organ-specific and non-organ-specific autoantibodies in patients with chronic HCV infection before and after IFNα therapy.

Patients and Methods: The prevalence of autoantibodies was investigated in 77 HCV-infected patients at baseline and after 24 weeks of treatment with peginterferon α2A. Investigated serum autoantibodies comprised antinuclear (ANA), anti-smooth muscle (ASMA), antimitochondrial (AMA), anti-parietal cell (PCA), antiperoxidase (anti-TPO), antithyreoglobulin (anti-TG), anticyclic citrullinated peptide (anti-CCP), rheumatoid factor (RF) IgM and IgA. Autoantibodies were detected by indirect immunofluorescence and the ELISA method. Patient characteristics included demographic, biochemical, serological, virological and liver histological factors.

Results: Baseline prevalence of autoantibodies was as follows: ANA were detected in 26% of samples, AMA in 3.9%, ASMA in 7.8%, PCAI in 7.8%, anti-TPO in 5.2%, anti-TG in 1.3%, anti-CCP in none, IgM RF in 83%, IgA RF in 83%. Interferon treatment resulted in significant changes in ANA (de novo induction in 6% and titre increase in 6%) and a significant decrease in IgM and IgA RF titres. No auto-CCP antibodies were induced. AMA synthesis was associated with liver activity score and AMA and ASMA with patients’ age. There was no association of autoantibody synthesis with viral load or liver histopathology findings. Apart from thyroid dysfunction in 15% of patients no clinically overt autoimmune diseases were observed.

Conclusion: Interferon therapy resulted in an increase in ANA synthesis and a decrease in RF levels. Thyroid dysfunction was observed in 15% of subjects, in the others changes in autoantibody titres were not clinically relevant.

PROTEIN BIOCHIP ARRAY TECHNOLOGY FOR CYTOKINE PROFILING PREDICTS ETANERCEPT RESPONSIVENESS IN RHEUMATOID ARTHRITIS

S Fabre, 1AM Dupuy, C Guisset, N Doussat, 1JP Cristol, 1JP Daures, C Jongensen. 1Immuno-rheumatology, Lapeyronie University Hospital, Montpellier, France; 2Biochemistry Laboratory, Lapeyronie University Hospital, Montpellier, France; 3Institut Universitaire de Recherche Clinique, Montpellier, France

In rheumatoid arthritis (RA) there are currently no good indicators to predict a clinical response to tumour necrosis factor alpha (TNFα) blockade. The purpose of this study was to determine the role of peripheral blood cytokine profiling in differentiating between a good versus a poor response to etanercept in RA.
Methods: Peripheral blood samples were collected at baseline and at 3 months from 33 patients with active disease who were treated twice weekly by etanercept therapy. Responders are defined by the presence of three out of four American College of Rheumatology criteria: 20% or greater decrease in C-reactive protein (CRP), visual analogue score of disease activity, erythrocyte sedimentation rate (ESR), and improvement of the disease activity score (DAS28; four values) by 1.2 or more obtained at 3 months. Twelve cytokines were measured from serum collected on days 0 and 90 by proteomic array (protein biochip array; Investigator Evidence, Randox, France) including IL-6, TNFα, IL-1α, IL-1β, IL-2, IL-8, IFNγ, IL-4, IL-10, monocyte chemoattractant protein type 1 (MCP-1), epidermal growth factor (EGF) and vascular endothelial growth factor. Our results showed that high serum levels of MCP-1 and EGF were associated with a response to etanercept. In addition, the increase of two combined parameters CRP and EGF was predictive of a response to etanercept treatment at 3 months (sensitivity 87.5% and specificity 75%, accuracy 84.4%).

Conclusion: These findings suggest that cytokine profiling by proteomic analysis before treatment initiation may help to identify a responder patient to TNFα blocking agents in RA.

A16 THE FC GAMMA IIB RECEPTOR DAMPENS TLR4-MEDIATED IMMUNE RESPONSES AND IS HIGHLY EXPRESSED ON DENDRITIC CELLS FROM RHEUMATOID ARTHRITIS PATIENTS WITH QUIESCENT DISEASE

M Wenink, M Roelofs, R Huijbers, H Koener, R van Beek, E Joosten, E Meyer-Wentrup, J Mathison, J Ronnfeldt, A Gemaera, E Borov, S Koeng, W van den Berg, P van Riel, T Radstake. Department of Rheumatology, Redbous University, Nijmegen Medical Centre, Nijmegen, The Netherlands; Department of Bloodtransfusion and Transplantation Immunology, Redbous University, Nijmegen Medical Centre, Nijmegen, The Netherlands; Tumor Immunology Laboratory, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands; Unit of Clinical Immunology, Uppsala University, Uppsala, Sweden, “MacroGenics Inc, Rockville, USA

Background and Objectives: Little is yet known about the exact pathogenesis of rheumatoid arthritis (RA), let alone the mechanisms at work aimed at dampening the chronic inflammatory response. In the present study we aimed to delineate whether RA patients who are able to discontinue antirheumatic treatment (disease-modifying antirheumatic drugs; DMARD® RA) successfully have phenotypically and functionally different dendritic cells (DC) compared with RA patients in need of antirheumatic drugs (DMARD® RA) and healthy controls.

Materials and Methods: 52 RA patients were selected from our well-documented prospective cohort. In addition, 10 healthy controls were included. All patients in our cohort who were not on DMARD® therapy for more than 2 years were selected and matched with RA patients in need of DMARD® therapy. Monocytes were isolated and cultured into DC. DC were analysed for their expression of DC markers and Fc gamma receptor (FcgR) I/IIa/IIb and III. DC from DMARD® RA and DMARD® RA patients and healthy controls were stimulated with immune complexes (IC) and/or lipopolysaccharide. The release of tumour necrosis factor alpha (TNFα) and IL-12p70 was determined. Allogeneic T cells were cultured with the DC for 6 days before stimulation with phorbol myristate acetate and ionomycin, after which the levels of IL-4, IL-15, IFNγ and IL-17 in the supernatants were determined. In addition, the amount of regulatory T cells was evaluated. For further analysis of the pathways involved we added various inhibitors for mediators known to play a role in these pathways before the stimulation with IC and/or lipopolysaccharide. In addition, we evaluated the phosphorylation of Shp and Akt and degradation of the inhibitor of nuclear factor kappa B isoform alpha (IκBα) in the various DC.

Results: DC from DMARD® RA patients expressed the inhibitory FcgRIIB at very high levels compared with DMARD® RA patients or healthy controls. The activating FcgR and all other markers were equally expressed in all groups. The expression of FcgRIIB on DC was negatively correlated with disease activity in DMARD® RA patients. We observed that only DC from DMARD® RA patients were able to inhibit pro-inflammatory TLR4 responses when co-stimulated with IC, decreasing the release of TNFα and IL-12p70 and dampening the ability to induce T-cell proliferation. In addition, the production of Th2 cytokines by T cells as well as the presence of regulatory T cells was markedly enhanced. By the use of blocking antibodies specific for FcgRIIa and FcgRIIB we demonstrated that FcgRIIB is crucial for the inhibitory effect of IC on TLR4 responses. The inhibitory effect of FcgRIIB is mediated via the PI3K/Akt pathway and is characterised by the increased phosphorylation of Shp and Akt and the decreased degradation of IκBα.

Conclusions: RA patients who are able to discontinue antirheumatic treatment successfully express highly increased levels of

A15 RAPAMYCIN-TREATED DENDRITIC CELLS FOR CELL-BASED IMMUNOMODULATORY THERAPY IN ARTHRITIS

J Quentin, L-M Charbonnier, C Jorgensen, P Louis-Plience. Inssem UB44, Montpellier, France

Background: Dendritic cells (DC) are professional antigen-presenting cells that have unique properties to steer the outcome of immune responses. We previously demonstrated that the repetitive injection of immature DC was able to protect mice from developing collagen-induced arthritis (CIA). Rapamycin is a macrolide antibiotic with potent immunosuppressive properties introduced in collagen-induced arthritis (CIA). Rapamycin is a macrolide antibiotic with potent immunosuppressive properties introduced in collagen-induced arthritis (CIA). Rapamycin can be used in the clinic for autoimmune diseases and cancer. Rapamycin-treated DC decreased the immunogenicity of immature DC and evaluated the tolerogenic potential of such rapamycin-treated immature DC. It could be explained by the increase of IL-10 and IL-12p70 and the decrease of IFNγ secreted by CD4+ cells in particular natural killer cells, observed at day 28 in the liver of immature DC rapamycin-treated mice.

Methods: Rapamycin-treated DC were generated in the presence of 10 ng/ml rapamycin during 6 days. The maturation of rapamycin-treated immature DC was induced or not by the addition of lipopolysaccharide. DC cytokine secretion profiles were quantified by ELISA and their phenotype monitored by FACS analysis. In in-vivo experiments, repetitive injections of 5 × 10⁶ rapamycin-treated DC were performed in naive mice and regulatory populations monitored in various lymphoid organs. In the CIA mouse model, repetitive injections of immature DC or rapamycin-treated DC were performed at days 21, 25 and 25 after immunisation.

Results: The phenotypes of rapamycin-treated immature DC and mature DC were slightly different from conventional immature and mature DC, with a decreased expression of CD40 and co-stimulatory molecules such as CD86. The increase in the immunogenicity of rapamycin-treated DC was confirmed in a mixed-lymphocyte reaction. In vivo, we observed an increase of the IL-10-secreting CD4+CD49b+ T lymphocytes in the liver and spleen of mice injected with rapamycin-treated DC. In experimental arthritis, repetitive injections of rapamycin-treated DC decreased the severity of established arthritis more importantly than conventional immature DC. It could be explained by the increase of IL-10-secreting CD4+CD49b+ and the decrease of IFNγ secreted by CD4+ positive and negative cells, in particular natural killer cells, observed at day 28 in the liver of immature DC rapamycin-treated mice.

Conclusion: Our results suggest that the tolerogenic potential of immature DC is increased when generated in the presence of rapamycin. The therapeutic potential of such a cell-based immunomodulatory strategy might be assessed in rheumatoid arthritis.
**A17** THE MINERAL OIL PRISTANE INDUCES APOPTOSIS IN ARTHRITIS-PRONE DA.1F RATS

S Herman, M Hoffmann, J Smolen, G Steiner. Department of Rheumatology, Medical University of Vienna, Vienna, Austria

**Objectives**: In recent years apoptosis has emerged to explain how self-antigens might become available to a self-primed immune system. Pristane (2,6,10,14-tetramethylpentadecane) is a defined mineral oil that elicits autoimmune disorders such as lupus-like disease or arthritis in experimental animal models and induces apoptosis in murine cell lines and peritoneal exudate cells. In susceptible rat strains, such as the DA.1F rat, arthritis is induced by intradermal or subcutaneous injection of pristane at the base of the tail. The injected oil has been shown to disseminate quickly and specifically to local lymph nodes after injection. We were therefore interested whether apoptosis is important for the pathogenesis of pristane-induced arthritis.

**Materials and Methods**: Female DA.1F rats were immunised intradermally with 200 µl pristane at the base of the tail. Draining inguinal lymph nodes were analysed on days 3, 9 and 14 after pristane application. Healthy DA.1F rats served as controls in all experiments. Cells from inguinal lymph nodes were isolated, stained with annexin V and 7AAD and FACS analysis was performed. Furthermore, lymph nodes were analysed by means of immunohistochemistry.

**Results**: FACS staining exhibited an annexin V positive plus 7AAD low population in lymph node cells from pristane-primed but not naive animals. Furthermore, immunohistochemical stainings revealed a significantly higher amount of TUNEL+ and cleaved caspase-3+ cells in lymph nodes that had been exposed to pristane.

**Conclusion**: The finding of an increased amount of apoptotic cells in draining lymph nodes presents a potential explanation for the mechanism by which pristane induces autoimmunity in DA.1F rats. Uptake of autoantigens provided by sustained apoptosis in the setting of an inflammatory milieu in the draining lymph nodes may lead to the enhanced presentation of autoantigens. A break in self-tolerance and the development of inflammatory arthritis might be the result.

**A18** THE SALIVARY GLAND EPITHELIAL CELLS OF PATIENTS WITH SJÖGREN’S SYNDROME DISPLAY REDUCED RESPONSIVENESS TO OESTROGENS

M Tsinti, E Kassi, HM Moutsopoulos, MN Manoussakis. Department of Pathophysiology, National and Kapodistrian University of Athens, Athens, Greece

**Background and Objectives**: Salivary gland epithelial cells (SGEC) appear to play an important role in the development of the inflammatory reactions that characterise primary Sjögren’s syndrome (SS). Oestrogens are possibly implicated in SS pathogenesis as suggested by the strong female preponderance of the disease. The possible role of oestrogen deprivation in SS pathophysiology has been supported by the induction of SS-like lesions in the salivary epithelium of oestrogen-depleted experimental animals. In this study, we used a functional in-vitro assay to evaluate comparatively the responsiveness to oestrogens of the SGEC of SS patients (SS-SGEC) and of disease control individuals (Ct-SGEC).

**Methods**: Minor salivary gland (MSG) biopsy specimens and cultured non-neoplastic SGEC lines established from MSG tissues were obtained from 14 SS patients and 12 disease controls (according to the American-European SS classification criteria). The responsiveness of SS-SGEC lines (n = 8) and Ct-SGEC lines (n = 12) to oestrogens was assessed by flow cytometry analysis of

**Results**: In recent years apoptosis has emerged to explain how self-antigens might become available to a self-primed immune system. Pristane (2,6,10,14-tetramethylpentadecane) is a defined mineral oil that elicits autoimmune disorders such as lupus-like disease or arthritis in experimental animal models and induces apoptosis in murine cell lines and peritoneal exudate cells. In susceptible rat strains, such as the DA.1F rat, arthritis is induced by intradermal or subcutaneous injection of pristane at the base of the tail. The injected oil has been shown to disseminate quickly and specifically to local lymph nodes after injection. We were therefore interested whether apoptosis is important for the pathogenesis of pristane-induced arthritis.

**Materials and Methods**: Female DA.1F rats were immunised intradermally with 200 µl pristane at the base of the tail. Draining inguinal lymph nodes were analysed on days 3, 9 and 14 after pristane application. Healthy DA.1F rats served as controls in all experiments. Cells from inguinal lymph nodes were isolated, stained with annexin V and 7AAD and FACS analysis was performed. Furthermore, lymph nodes were analysed by means of immunohistochemistry.

**Results**: FACS staining exhibited an annexin V positive plus 7AAD low population in lymph node cells from pristane-primed but not naive animals. Furthermore, immunohistochemical stainings revealed a significantly higher amount of TUNEL+ and cleaved caspase-3+ cells in lymph nodes that had been exposed to pristane.

**Conclusion**: The finding of an increased amount of apoptotic cells in draining lymph nodes presents a potential explanation for the mechanism by which pristane induces autoimmunity in DA.1F rats. Uptake of autoantigens provided by sustained apoptosis in the setting of an inflammatory milieu in the draining lymph nodes may lead to the enhanced presentation of autoantigens. A break in self-tolerance and the development of inflammatory arthritis might be the result.

**A19** TLR2 PROMOTES TH2/TH17 RESPONSES VIA TLR4 AND TLR8 BY HUMAN DENDRITIC CELLS BY ABDROGGING THE TYPE 1 INTERFERON AMPLIFICATION LOOP

M Wenink, E Kassenge, S Abdallah-Boudaoud, I van Bon, J Broen, C Popa, R Huijbens, E Lubberts, P van Riel, W van den Berg, T Radstake. Department of Rheumatology, Radboud University, Nijmegen Medical Center, Nijmegen, The Netherlands; 2Department of Rheumatology, Erasmus Medical Center, Rotterdam, The Netherlands

**Background and Objectives**: Whereas the function of most Toll-like receptors (TLR) seems clearly aimed at removing invading bacteria and viruses, the precise function of TLR2 is less clear. We aimed to delineate the role of TLR2 in the modification of the phenotype and functionality of human dendritic cells (DC) and its interaction with other TLR.

**Materials and Methods**: Monocyte-derived DC were cultured from 15 healthy controls and stimulated with various TLR ligands in combination with Pam2Cys (P2C, TLR2/6) or Pam3Cys (P3C, TLR2/1) or alone. As a readout, IL-6, IL-10, tumour necrosis factor alpha (TNFα), IL-12p70, IL-23, IL-1α, IL-1β and monocyte chemotactic protein type 1 (MCP-1) were measured and mixed leucocyte reactions (MLR) were performed. In additional MLR experiments IL-1ra or antibodies neutralising IL-6R, OX40L, transforming growth factor beta, IL-12p35 or IL-12p40 were added. To delineate further the pathways involved the expression of interferon regulatory factors (IRF) 1, 7 and 8, Jagged-1, SOCS1, SOCS3 and A20 were examined as well as the phosphorylation of STAT1. In addition, the involvement of IFNα, IFNβ and IL-10 was evaluated.

**Results**: Both P3C and P2C dose-dependently inhibited lipopolysaccharide (TLR4) and R848 (TLR8) induced cytokine production, particularly IL-12p70, but not flagellin (TLR5) and even enhanced Poly(I : C) (TLR3) mediated cytokine production. Only the release of MCP-1 was increased by the presence of TLR2 ligands. We found that TLR2 inhibits the type 1 interferon amplification loop of TLR4

**Conclusion**: This study indicates that SGEC derived from SS patients display reduced responsiveness to oestrogens, compared with controls, a fact whose pathogenetic implication needs to be elucidated. The defective oestrogenic responsiveness of SS epithelia does not appear to be caused by the altered expression of ER proteins and may represent a manifestation of the intrinsic activation that characterises the epithelial cells of SS.
and TLR8 by abrogating the production of type 1 interferons, inhibiting the phosphorylation of STAT1 and strongly reducing the transcription of IRF1 and IRF8. In line with this, an increased expression of SOCS1 was observed. Furthermore, the inhibitory effect of TLR2 on the release of TNFα but not of IL-12p70 was mediated by PI3K. Erk or IL-10 did not play a role in the TLR2-mediated suppression of TLR4/8 responses. Concomitant stimulation with TLR2 ligands did not influence TLR4/8 mediated phenotypic maturation of DC or their ability to induce T-cell proliferation. TLR2 co-activation did lead to a significant shift from Th1 to Th2 and Th17 prone responses. This was especially clear when DC were triggered with the potent synergistic combination of lipopolysaccharide and R848, which was inherent in the inability of TLR2 co-stimulated DC to produce IL-12p70. We found that IL-6 and IL-1 were essential in DC-facilitated Th17 differentiation.

Conclusions: Here we report that TLR2 was able to dampen the TLR4 and TLR6-induced cytokine production by DC and led to a shift from Th1 to Th2 and Th17 cell differentiation. TLR2 activation led to an abrogation of the type 1 interferon amplification loop. Collectively, we provide a novel mechanism by which TLR2 dampens TLR signalling and subsequently skews the immune response from a Th1 towards a Th2/Th17 response. This puts TLR2 in the middle of the immune network deciding whether the effector response against microorganisms or in autoimmunity is mainly Th1 or Th17 mediated.

A20 TUMOUR NECROSIS FACTOR BLOCKADE IMPAIRS T-CELL-DEPENDENT ANTIBODY RESPONSES

GF Salinas, TL De Rycke, TP Ramans, M van den Burg, B Barendregt, PP Tak, BD Baeten. Department of Clinical Immunology and Rheumatology, Academic Medical Center, Amsterdam, The Netherlands; Department of Immunology, Erasmus Medical Center, Rotterdam, The Netherlands

The first two authors contributed equally to this work.

Objective: Antinuclear antibodies induced by tumour necrosis factor (TNF) blockade are restricted to the IgM isotype and mainly directed towards T-cell-independent (TI) antigens. As this suggests that TNF blockade impairs the maturation of the B-cell responses towards T-cell-dependent (TD) antigens, we investigated the humoral response to vaccination during TNF blockade in spondyloarthritis.

Materials and Methods: 30 spondyloarthritics patients were vaccinated with energeix, a TD vaccine to hepatitis B, and with pneumovax, a TI vaccine to the polysaccharide of Streptococcus pneumoniae. Of these, 10 were untreated (control group) and 20 were treated with TNF blockade. A second cohort of 10 spondyloarthritics patients treated with infliximab was vaccinated with prevnar, a TD vaccine to S pneumoniae. Serum and peripheral blood lymphocytes were collected before and up to 26 weeks after vaccination. Vaccine-specific antibody titres were determined by ELISA. Somatic hypermutation was assessed by the Igk REHMA assay (Anderson, 2005). The phenotype and expression of co-stimulatory molecules were evaluated by flow cytometry.

Results: The control group displayed clear IgM (230 mU/ml, 130–397) and IgG (3300 mU/ml, 1515–4785) responses against the TI vaccine pneumovax. Both IgM (69 mU/ml, 25–131, p = 0.02) and IgG responses (1545 mU/ml, 729–2560, p = 0.01) were moderately but significantly decreased in anti-TNF-treated patients. For the TD vaccine energeix, IgG responses were clearly present in the controls (595 IU/ml, 73–1750), but almost absent in treated patients (10 IU/ml, 0–112, p = 0.006). The more profound suppression of TD versus TI responses by TNF blockade was confirmed by the anti-S pneumoniae IgG induced by prevnar (485 mU/ml, 143–1210) in comparison with pneumovax (1545 mU/ml, 729–2560) in infliximab-treated patients (p = 0.05). Accordingly, there was a trend towards decreased somatic hypermutation in the total B-cell population after infliximab treatment (mean decrease 6.01%, p = 0.07). Investigating the underlying mechanisms, phenotypic analysis of circulating B (naive, activated, memory, plasmablasts) and T (CD3, CD4, CD8) lymphocytes did not reveal significant changes during TNF blockade. However, infliximab significantly decreased CD8 expression on B cells (p = 0.003 at week 12 and p = 0.002 at week 22) and CD27 expression on CD4 (p = 0.02 at week 12 and p = 0.001 at week 22) and CD8 (p = 0.02 at week 12 and p = 0.006 at week 22) T cells. Moreover, infliximab also decreased the CD28 expression by CD8 T cells at week 22 (p = 0.04).

Conclusion: TNF blockade severely impairs the induction of TD humoral responses. The contribution of the downregulation of CD8, which modulates the B-cell receptor signal and the co-stimulatory molecules CD27 and CD28 is currently under investigation.

A21 T-REGULATORY CELLS: T-CELL REGULATORS OF BONE

1MM Zaiss, TK Sarier, B Frey, A Hess, J Zweimüller, K Engeli, G Kiakia, LE Douin, R Volt, M Burkhardt, R Rauch, R Kröckel, G Ziegler, G Schett, UP Davids. Department of Internal Medicine 3, Rheumatology and Immunology, University of Erlangen-Nuremberg, Erlangen, Germany; 2Department of Radiation Oncology, University of Erlangen-Nuremberg, Erlangen, Germany; 3Institute of Experimental and Clinical Pharmacology and Toxicology, University of Erlangen-Nuremberg, Erlangen, Germany; 4Institute of Medical Physics, University of Erlangen-Nuremberg, Erlangen, Germany; 5Institute of Immunology, Alexander Fleming Biomedical Sciences Research Center, Van, Greece; 6SCANCO Medical AG, Brütisellen, Switzerland; 7Department of Paediatrics, University of Erlangen-Nuremberg, Erlangen, Germany; 8Molecular Immunology, Robert Koch-Institute, Berlin, Germany; 9Benaroya Research Institute, Virginia Mason, Seattle, Washington, USA

Background and Objectives: Immune activation as seen in inflammatory arthritis triggers bone loss. Whether immune regulation actively protects bone is unclear. However, we recently demonstrated that T regulatory cells (Treg) can block the differentiation of the bone resorbing cells or osteoclasts in vitro. This inhibition was shown to require cell-to-cell contact and to be mediated via CTLA-4. We therefore address the role of Treg in controlling normal bone homeostasis as well as rheumatoid arthritis-induced bone destruction in vivo.

Materials and Methods: The bone parameters of mice with increased numbers of Treg (foxp3-transgenic mice) were compared with their wild-type littermates. Ovariectomy was performed as a model for post-menopausal osteoporosis. Bone marrow isolated from foxp3-transgenic mice or from foxp3-deficient mice that are lacking Treg were transferred into human tumour necrosis factor (TNF) alpha transgenic mice (hTNFtg mice) to address the function of Treg in arthritis-mediated bone destruction. The bones of mice deficient for co-stimulatory T-cell receptor molecules known to be involved in Treg activity were phenotyped to mechanistically analysed Treg function in bone. Treg isolated from the various mice were co-cultured with monocytes in order to determine their capability to suppress osteoclastogenesis in vitro.

Results: We showed that mice with increased Treg (foxp3-transgenic mice) developed high bone mass and were protected from ovariectomy-induced osteoporosis, inflammatory osteopenia and arthritic bone destruction, whereas foxp3-deficiency enhanced TNF-induced bone loss. The skeletal effects of Foxp3 were mediated through inhibition of osteoclast-mediated bone resorption. Binding of Treg to CD80/CD86 on osteoclast precursors was essential for inhibiting osteoclast differentiation. Importantly, CD80/86-deficient mice developed osteopenia as a result of increased osteoclast differentiation, whereas other co-stimulation mutants did not show any bone phenotype. Engaging of CD80/86 by Treg through CTLA-4 induced indoleamine 2,3-dioxygenase expression in osteoclast precursors increasing tryptophan catabolism and increasing the rate of apoptosis.

Conclusions: These results demonstrate that Foxp3-expressing Treg control bone resorption and preserve bone mass during physiologic and pathological bone remodelling.

1Department of Clinical Immunology and Rheumatology, Academic Medical Center, Amsterdam, The Netherlands; 2Department of Immunology, Erasmus Medical Center, Rotterdam, The Netherlands; 3Department of Clinical Immunology and Rheumatology, Academic Medical Center, Amsterdam, The Netherlands; 4Department of Immunology, Erasmus Medical Center, Rotterdam, The Netherlands; 5Department of Rheumatology, University of Erlangen-Nuremberg, Erlangen, Germany; 6SCANCO Medical AG, Brütisellen, Switzerland; 7Department of Paediatrics, University of Erlangen-Nuremberg, Erlangen, Germany; 8Molecular Immunology, Robert Koch-Institute, Berlin, Germany; 9Benaroya Research Institute, Virginia Mason, Seattle, Washington, USA
2. Genetics, epigenetics and environmental factors

A22  A GENETIC MODEL FOR IRF5, AUTOANTIBODIES AND INTERFERON IN THE PATHOGENESIS OF SYSTEMIC LUPUS ERYTHEMATOSUS

1TB Newbold, 2JA Kelly, 3SN Karuki, 4K Thomas, 5D Walker, 6S Kamp, 7AK Wong, 8JT Merrill, 2-7ME Alarcón-Riquelme, 8-10RG Ramsey-Goldman, 7-10JD Reveille, 11MA Petri, 9-10JC Edberg, 11RPR Kimberly, 9-10GS Alarcón, 11T Vyse, 2-4JA James, 9PM Gaffney, 8KL Moser, 2-8MK Crow, 2-8,13JB Harley, 1Section of Rheumatology, University of Chicago, Chicago, Illinois, USA; 2Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA; 3MRC Unit for Longlife, Health and Ageing, London, UK; 4Department of Medicine, University of Oklahoma, Oklahoma City, Oklahoma, USA; 5University of Uppsala, Uppsala, Sweden; 6Division of Rheumatology, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA; 7Division of Rheumatology, University of Texas-Houston Health Science Center, Houston, Texas, USA; 8Division of Rheumatology, Johns Hopkins University, Baltimore, Maryland, USA; 9University of Alabama at Birmingham, Birmingham, Alabama, USA, 10PROFILE Group, 11Imperial College, London, UK, 12Mary Kirkland Center for Lupus Research, Hospital for Special Surgery, New York, New York, USA; 13US Department of Veteran Affairs Medical Center, Oklahoma City, Oklahoma, USA.

Background: High serum interferon alpha (IFNa) activity is implicated as a heritable risk factor for systemic lupus erythematosus (SLE). Interferon regulatory factor 5 (IRF5) functions downstream of endosomal Toll-like receptors, and its genetic variants have been associated with SLE susceptibility. SLE-specific autoantibodies in immune complexes may directly contribute to disease pathogenesis by linking specific IRF5 variants to IFNa production through the Toll-like receptor pathway.

Methods: IRF5 genotypes and serum autoantibodies were determined in 1034 SLE patients, 969 controls, 66 healthy first-degree relatives of European ancestry and in 456 SLE patients and 680 controls of African-American ancestry. Serum IFNa activity was measured using a functional assay.

Results: In European ancestry SLE patients, anti-dsDNA and anti-Ro autoantibodies were each associated with different IRF5 SLE risk alleles. Other autoantibodies were not associated. The risk of SLE as a result of IRF5 was largely dependent upon those subjects with anti-dsDNA and anti-Ro autoantibodies (p = 7.9 × 10^-16) for association of IRF5 with SLE in 393 subjects with these antibodies, p = 0.0019 in 641 patients lacking these antibodies, 70.4% of IRF5 attributable risk caused by subjects with anti-dsDNA or anti-Ro). The risk of SLE because of IRF5 in African-American ancestry subjects was also dependent upon anti-dsDNA and anti-Ro antibodies. In both ancestral backgrounds, IRF5 SLE risk variants resulted in increased serum IFNa only in those patients with either anti-dsDNA or anti-Ro antibodies. Healthy first-degree relatives who typically lack these autoantibodies demonstrated no relationship between the IRF5 genotype and serum IFNa.

Conclusions: Both increased serum IFNa and SLE risk due to the IRF5 genotype are largely dependent upon SLE-specific autoantibodies. These data suggest a disease model in which autoantibodies are directly pathogenic, cooperating with IRF5 variants to result in the dysregulation of IFNa production and the subsequent risk of SLE.

A23 CITTIRULINATED ALPHA-ENOLASE: "THE SMOKING GUN" IN RHEUMATOID ARTHRITIS

1H Mahdi, 2B Fisher, 3H Kalberg, 4D Plant, 4V Malmstrom, 5J Ronnelid, 6P Charles, 7B Ding, 1L Alfredsson, 1L Padyukov, 4D Symmons, 5P Venable, 1L Klareskog, 5K Lundberg, 1Rheumatology Unit, Karolinska Institute, Stockholm, Sweden; 2Kennedy Institute of Rheumatology, Imperial College London, London, UK, 3Institute for Environmental Medicine, Karolinska Institutet, Stockholm, Sweden; 4Arthritis Research Campaign Epidemiology Unit, University of Manchester, Manchester, UK; 5Unit of Clinical Immunology, Uppsala University, Uppsala, Sweden

Background and Objectives: In rheumatoid arthritis (RA), there is an association between smoking, HLA-DRB1 shared epitope (SE) and antibodies to cyclic citrullinated peptides (anti-CCP). However, CCP do not share sequence homology with any known human protein, and when elucidating the aetiology of RA it is essential to study true physiological autoantigens. Cittirullinated α-enolase is present in the rheumatoid joint and antibodies to the immunodominant epitope CEP-1 are present in 37–62% of patients, but only 3% of controls. In the present study, we examine the relationship between established genetic and environmental risk factors for RA and autoimmunity to the novel autoantigen CEP-1.

Materials and Methods: We studied three RA cohorts; the Epidemiological Investigation of RA (1000 RA patients, 872 healthy controls), the National Repository (279 RA patients) and the Norfolk Arthritis Register (218 RA patients). Anti-CCP and anti-CEP-1 antibody status was assayed by ELISA; genotyping (HLA-DRB1, FFTP22 and 2221 single nucleotide polymorphisms (SNP) across the major histocompatibility complex region) by standard PCR methods and information about smoking by questionnaire. Odds ratios (OR) for developing RA associated with each of the risk factors were calculated together with 95% CI, by means of logistic regression models.

Results: We could identify three major (CEP-1+/CCP+, CEP-1-/ CCP+, CEP-1+CCP+) and one minor (CEP-1+/CCP−) disease subsets. When comparing the three major, we found that HLA-DRB1*01 was mainly associated with CEP-1+/CCP− double-positive patients. This association was primarily with DR4, not DR1 alleles. The HLA-DRB1*01 smoking interaction also segregated with the CEP-1-positive subset. Here, a striking OR of 53 was demonstrated for smokers, homozygous for the SE, which should be compared with an OR of 6 for the corresponding CEP-1−/CCP+ subset. In addition, the HLA-DRB1*01−SE−FFTP22 interaction conferred a higher risk for the CEP-1+/CCP+ population (OR 17.8), compared with the CEP-1−/CCP+ (OR 4.6). Finally, 299 disease-associated SNP, across the entire major histocompatibility complex, were also preferentially linked to the CEP-1-positive subset of patients.

Conclusion: Our epidemiological investigation shows that major genetic and environmental susceptibility factors for RA, previously described in CCP-positive disease, are associated mainly with the anti-CEP-1-positive subset. Specific SE alleles, *0401 and *0404, as well as smoking, can now be linked with an autoimmune response to a physiological autoantigen, ctitrullinated α-enolase. We conclude that these susceptibility factors constitute a risk mainly in conjunction with immunity to CEP-1, rather than CCP. Therefore, in a substantial proportion of RA patients, ctitrullinated α-enolase may be a key antigen of aetiological importance.

A24 EFFECTS OF FC-GAMMA RECEPTOR COPY NUMBER VARIATION AND POLYMORPHISMS ON RESPONSE TO ADALIMUMAB IN RHEUMATOID ARTHRITIS PATIENTS

1-10WB Breunis, 2PL Klarenbeek, 3MV Herenius, 4CA Wijbrands, 5J Geissler, 6G Woltink, 6H van der Horst-Bruinsma, 7UBA Crusis, 8MTI Nourmohamed, 9BAC Dijkmans, 9PP Tak, 9N de Vries, 10TW Kuipers. 1Department of Pediatric Hematology, Hematology and Infectious Disease, Emma Children's Hospital, AMC/University of Amsterdam, Amsterdam, The Netherlands; 2Department of Clinical Immunology and Rheumatology, Amsterdam, The Netherlands; 3Department of Blood Cell Research and Experimental Immunohematology, Sanquin Research and Landsteiner Laboratory, Amsterdam, The Netherlands; 4Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Amsterdam, The Netherlands; 5Department of Rheumatology, VU University Medical Center, Amsterdam, The Netherlands; 6Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands; 7Department of Rheumatology, Jan van Breemen Institute, Amsterdam, The Netherlands.

Background and Objectives: Single nucleotide polymorphisms (SNP) in Fc-gamma receptors (FcgR) have been implicated in susceptibility for rheumatoid arthritis (RA) and in response to treatment with “biologics”. So far, studies reported conflicting results on both subjects. Several factors, such as homology between the receptor genes and copy number variation (CNV), might have
contributed to the conflicting results. In this study we used a recently developed multiplex ligation-dependent probe amplification (MLPA) assay to analyse both CNV and SNP in the FcgR region in RA patients. We investigated susceptibility for RA and response to treatment with adalimumab.

Methods: 97 white Dutch RA patients were included who had active disease despite treatment with disease-modifying antirheumatic drugs. Patients were started on treatment with adalimumab (40 mg per 2 weeks). Disease activity was evaluated by the disease activity score (DAS28) at baseline, after 4 weeks and after 16 weeks of therapy. 129 white Dutch healthy volunteers served as the control population. The MLPA assay was performed for the following FcgR-encoding genes (and functional SNP): FCGR2A(131H/R), FCGR2B(232I/T), FCGR2C(exon3 ORF/STOP), promoter region of FCGR2B/C(−386G/C), FCGR3A(158V/F), FCGR3B(HNA1a/HNA1b/HNA1c).

Results: RA susceptibility: CNV was observed for the genes encoding FcgR2C, 5A, and 5B in both patients and controls. CNV frequencies were not significantly different among both groups. None of the analysed SNP showed significant differences in allele frequencies or genotypes between RA patients and controls. After stratification for anti-cyclic citrullinated peptide (aCCP) positivity continued investigation of the Fc gamma receptors, as downstream autoantibody and immune complex ligands, in the pathogenesis of RA and further evaluation as potential therapeutic targets.

Conclusions: We have provided further data to support the association of the FcgR genetic locus with “susceptibility” to RA, particularly in the autoantibody-positive subgroup. This supports the continued investigation of the Fc gamma receptors, as downstream autoantibody and immune complex ligands, in the pathogenesis of RA and further evaluation as potential therapeutic targets.

FCGR3A is associated with autoantibody-positive rheumatoid arthritis in a large white UK population

1. J Robinson, 1D Cooper, 1J Barrett, 1M Naven, 1D Corscadden, 1A Barton, 1A Wilson, 1P Emerly, 1J Isaacs, 1A Morgan. Leeds Institute of Molecular Medicine, University of Leeds, Leeds, UK; 2YEAR Consortium; 3Biologics in Rheumatoid Arthritis Genetics and Genomics Study Syndicate; 4aEpidemiology Unit, University of Manchester, Manchester, UK; 5School of Medicine and Biomedical Sciences, University of Sheffield, Sheffield, UK; 6Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK

Background: We have previously demonstrated that rheumatoid arthritis (RA) is associated with the higher affinity FCGR3A-158V allele in a modestly sized white UK population of 829 cases and 418 controls. Following recent genome-wide association studies, many new RA genetic loci have been identified and subsequently replicated in large DNA collections. In this study we have significantly expanded the size of our cohort and further explored the association between FCGR3A and autoantibody (rheumatoid factor (RF) and cyclic citrullinated peptide (CCP)) positive and negative RA.

Methods: The FCGR3A-158V/F polymorphism was genotyped by either direct sequencing or using a well-established SSCP assay in a total of 2967 white RA subjects and 1732 healthy white controls. Additional phenotypic data comprising RF and CCP status (n = 2655 and 993, respectively) and the presence or absence of articular erosions of the hands or feet on plain radiographs (n = 1872) were available.

Results: In the total cohort, the FCGR3A-158V allele was associated with RA when analysed under a recessive model (odds ratio (OR) 1.3, 95% CI 1.0 to 1.5, p = 0.03) and, in particular, RF-positive (OR 1.3, 95% CI 1.0 to 1.5, p = 0.03) and CCP-positive (OR 1.3, 95% CI 1.0 to 1.6, p = 0.03) RA. No significant association with articular erosion was observed in this cross-sectional cohort of variable disease duration.

Conclusions: We have provided further data to support the association of the FcgR genetic locus with “susceptibility” to RA, particularly in the autoantibody-positive subgroup. This supports the continued investigation of the Fc gamma receptors, as downstream autoantibody and immune complex ligands, in the pathogenesis of RA and further evaluation as potential therapeutic targets.

A26 GENE EXPRESSION AND REGULATION IN HUMAN SYSTEMIC LUPUS ERYTHEMATOSUS: A CLINICOPATHOLOGICAL STUDY USING MICRO RNA

1E Stagakis, ’M Nakou, ’G Bertias, ’E Choustoulaki, ’H Kritikos, ’D Eilpoulos, ’D Boumpas. Laboratory of Autoimmunity and Inflammation, Rheumatology, Clinical Immunology and Allergy, Heraklion, Greece; ’Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA

Background and Objectives: Micro RNA (miRNA) are a highly conserved class of small non-coding RNA that have recently emerged as potent negative regulators of gene expression. MiRNA regulate major biological processes and play a pivotal role in the regulation of both the innate and the adaptive immune system. The aim of this study was to investigate the role of this new class of genes in the immune response in systemic lupus erythematosus (SLE) and assess their putative role as disease biomarkers.

Materials and Methods: We isolated peripheral blood mononuclear cells (PBMC) and T and B lymphocytes from 34 SLE patients, 18 with active (SLEDAI >8) and 16 with inactive (SLEDAI <8) disease, compared with 20 healthy controls. RNA extraction was performed and 365 miRNA were tested by using miRNA microarray technology. Three different bioinformatic algorithms were used for the identification of the gene targets of selected miRNA that were found differentially expressed in SLE patients. Validation of the expression levels of those miRNA/gene target pairs was performed by real-time PCR in PBMC of SLE patients. Western blot analysis of the same samples was used to confirm the inverse correlation of miRNA and their protein targets.

Results: A total of 25 miRNA was differentially expressed between normal and SLE patients. Among them, 14 miRNA were differentially expressed between SLE patients with active versus inactive disease with seven miRNA found to be downregulated and seven upregulated. Among them, four miRNA (miR-21, miR-25, miR-106b, miR-148b) highly correlated with SLE disease activity (r2 more than 0.84). When T and B cells were analysed separately, miR-25, miR-143 and miR-106b in T cells and miR-25, miR-106b and miR-155 in B cells correlated with disease activity (r2 more than 0.94). Using bioinformatic algorithms, we identified that miR-16 regulates the expression of the antiapoptotic protein Bcl2, whereas miR-25 the proapoptotic protein Bim, shown to be involved in autoimmunity and lymphoproliferation in mice. Preliminary results demonstrate an inverse correlation of miRNA/protein targets expression in the miRNA level, as well as in the protein level.

Conclusions: Our work revealed a unique miRNA gene signature in human SLE with initial results confirming the involvement of apoptosis in the pathogenesis of the disease. Longitudinal data are needed to document further the role of miRNA as disease biomarkers.
NEW RHEUMATOID ARTHRITIS GENETIC FACTOR AND C5 SERUM LEVEL

1H Mbaraek, 2M Kallel-Sellami, 3Y Zerzi, 3K Dawidowicz, 3K Meji, 4T Bardin, 4B Prum, 5P Dieude, 6E Petit-Teoixea, 6F Cornelis, 7S Makni. 1Gh-Hotel EA3886, UniversitévEvry-Val d’Essonne, Paris, France; 2Laboratoire d’Immunologie, Hôpital la Rabita, 1007 Tunis, Tunisia; 3Département de Rhumatologie, Hôpital Lanbœsière, AP-HP, Paris, France; 4Laboratoire Statistique et Genomique, Evry, France; 5Centre Hospitalier Sud Francilien, Corbeil-Essonnes, France; 6Unité de Généétique Clinique, Hôpital Lanbœsière, AP-HP, Paris, France

Background: There is a significant body of evidence suggesting that uncontrolled complement activation plays an important role in the maintenance of the polyarticular chronic synovitis characteristic of rheumatoid arthritis (RA). Recently, a novel RA genetic risk factor was identified, involving allelic polymorphisms on chromosome 9, in the intergenic region between C5 (complement factor 5) and TRAF1 (tumour necrosis factor receptor-associated factor 1) genes. The new genetic factor, detected in RA case-control studies in north American, Swedish and Dutch populations, was confirmed by linkage in a family-based study in the white European population.

Objectives: We aimed at investigating whether this new C5-TRAF1 genetic factor would correlate with a higher C5 serum level in RA patients.

Methods: We measured C5 serum concentration in 194 rheumatoid factor-positive RA patients. The susceptibility genotype was present in 126 individuals: 60 homozygous patients for the most strongly RA-associated allele (rs10818488_A) and 66 heterozygous patients (rs10818488_AG). The protective genotype, homozygosity for the non-risk allele (rs10818488_G), was present in 68 patients. The C5 serum concentration was measured with a double-ligand ELISA using a rabbit polyclonal anti-human C5 antibody.

Results: The results showed no significant difference between the group of the susceptibility genotype (AA + AG) and the group of the protective genotype (GG) (p = 0.455). However, surprisingly, we observed a significantly lower C5 serum level in the homozygous AA group (mean 12%) compared with the heterozygous AG group (mean 15%, p = 0.034) and the protective GG group (mean 135%, p = 0.087).

Conclusions: These results suggest that, out of the two RA-associated genotypes, surprisingly, only the homozygous genotype for the risk allele (AA) might be correlated with a lower C5 serum level, but in the opposite direction from expectations. If replicated, this observation will have to be taken into account when investigating C5-TRAF1 RA susceptibility.

HISTONE DEACETYLASE INHIBITORS SUPPRESS INFLAMMATORY AND ANGIOTENIC CYTOKINE PRODUCTION BY RHEUMATOID ARTHRITIS SYNOVIAL MACROPHAGES, FIBROBLAST-LIKE SYNOVIOCYTES AND SYNOVIAL BIOPSY EXPLANTS

1AM Grabiec, 1NE Sanders, 2VD de Jager, 1BJPrakken, 1T Burakowski, 1WMalinski, 1PP Tak, 1KA Reuqoost. 1Division of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; 2Department of Pathophysiology and Immunology, Institute of Rheumatology, Warsaw, Poland

Background and Objectives: The accumulation and persistence of activated fibroblast-like synoviocytes (FLS) and macrophages contributes significantly to the pathology of rheumatoid arthritis (RA). Under inflammatory conditions, signalling pathways responsible for cellular activation are tightly regulated by the reversible acetylation and deacetylation of histones, transcription factors and structural proteins. Histone deacetylase (HDAC) inhibitors have demonstrated potent therapeutic effects in animal models of arthritis and other chronic inflammatory disorders. The purpose of this study was to examine the effects of HDAC inhibitors on the activation status of RA synovial macrophages and FLS, and intact RA synovial tissue.

Materials and Methods: Macrophages differentiated from RA synovial fluid (SF)-derived monocytes, RA FLS, and synovial tissue explants obtained from the clinically active joints of three RA patients by joint needle arthroscopy were treated with tumour necrosis factor alpha (TNFα), IL-1β, or lipopolysaccharide in the absence or presence of the HDAC inhibitor trichostatin A (TSA), suberyol-bis-hydroxycocid (SBHA), sodium phenylbutyrate, or nicotinamide. IL-6 and IL-8 production was measured by ELISA or intracellular staining and FACS analysis. Synovial explant production of chemokines, cytokines and growth factors known or implicated in perpetual inflammation in RA was determined by luminex analysis. Cytokine messenger RNA expression was assessed by semi-quantitative PCR, and acetylation of intracellular proteins was studied by immunoblotting.

Results: Each HDAC inhibitor, irrespective of their chemical class, potently and significantly blocked lipopolysaccharide and TNFα-induced IL-6 production by RA SF macrophages (p<0.01), while leaving IL-8 production unaffected. IL-6 production was inhibited at the level of gene transcription, and was more closely associated with acetylation of non-histone, rather than histone, proteins. In contrast, both TSA and nicotinamide (p<0.01), but not SBHA, suppressed TNFα-induced IL-8 production by RA FLS. All HDAC inhibitors suppressed RA FLS IL-8 production (p<0.01).

Conclusions: We demonstrated that inhibition of HDAC activity in RA synovial tissue and RA synovial cell populations efficiently blocks the production of multiple inflammatory cytokines, chemokines and angiogenic factors. Therapies targeting HDAC activity may be useful in suppressing inflammation and neovascularisation in RA.

RE-EVALUATION OF THE INTERACTION BETWEEN HLA-DRB1, PTPN22 AND SMOKING IN DETERMINING THE SUSCEPTIBILITY TO AUTOANTIBODY-POSITIVE AND NEGATIVE RHEUMATOID ARTHRITIS IN A LARGE UK WHITE POPULATION

1,2AW Morgan, 3,4IW Thomson, 3,5SG Martin, 1,2AM Carter, 1,3HA Ehrlich, 1Albott, 1LHocking, 1OM Reid, 1HP Harrison, 1WP Wordsworth, 1S Steer, 1JW Johnston, 1P Emery, 1AG Wilson, 1JH Barrett. 1Leeds Institute of Molecular Medicine, University of Leeds, Leeds, UK; 2,3Immunology Unit, University of Manchester, Manchester, UK; 3,5YEAR Consortium; 4Academic Unit of Molecular Vascular Medicine, The Light Laboratories, University of Leeds, Leeds, UK; 4UKRAK Consortium; 4Roche Molecular Systems, Pleasanton, California, USA; 4Bone Research Group, Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen, UK; 4University of Oxford Institute of Musculoskeletal Sciences, Botnar Research Centre, Oxford, UK; 4Clinical and Academic Rheumatology, Kings College Hospital NHS Foundation Trust, London, UK; 4School of Medicine and Biomedical Sciences, University of Sheffield, Sheffield, UK

Background and Objectives: The objective of this study was to define interactions between HLA-DRB1, PTPN22 and smoking in anti-cyclic citrullinated peptide (CCP) and rheumatoid factor (RF) positive and negative rheumatoid arthritis (RA).

Methods: Data from 5020 RA subjects and 3759 healthy controls recruited from six centres across the UK were analysed. The magnitude of association for each genotype and smoking were undertaken using additive and multiplicative models following adjustment for year of birth using Mantel–Haenszel methods. The results showed no significant difference between the group of the susceptibility genotype (AA + AG) and the group of the protective genotype (GG) (p = 0.455). However, surprisingly, we observed a significantly lower C5 serum level in the homozygous AA group (mean 12%) compared with the heterozygous AG group (mean 15%, p = 0.034) and the protective GG group (mean 135%, p = 0.087).

Conclusions: These results suggest that, out of the two RA-associated genotypes, surprisingly, only the homozygous genotype for the risk allele (AA) might be correlated with a lower C5 serum level, but in the opposite direction from expectations. If replicated, this observation will have to be taken into account when investigating C5-TRAF1 RA susceptibility.

HISTONE DEACETYLASE INHIBITORS SUPPRESS INFLAMMATORY AND ANGIOTENIC CYTOKINE PRODUCTION BY RHEUMATOID ARTHRITIS SYNOVIAL MACROPHAGES, FIBROBLAST-LIKE SYNOVIOCYTES AND SYNOVIAL BIOPSY EXPLANTS

1,2AW Morgan, 3,4IW Thomson, 3,5SG Martin, 1,2AM Carter, 1,3HA Ehrlich, 1Albott, 1LHocking, 1OM Reid, 1HP Harrison, 1WP Wordsworth, 1S Steer, 1JW Johnston, 1P Emery, 1AG Wilson, 1JH Barrett. 1Leeds Institute of Molecular Medicine, University of Leeds, Leeds, UK; 2,3Immunology Unit, University of Manchester, Manchester, UK; 3,5YEAR Consortium; 4Academic Unit of Molecular Vascular Medicine, The Light Laboratories, University of Leeds, Leeds, UK; 4UKRAK Consortium; 4Roche Molecular Systems, Pleasanton, California, USA; 4Bone Research Group, Department of Medicine and Therapeutics, University of Aberdeen, Aberdeen, UK; 4University of Oxford Institute of Musculoskeletal Sciences, Botnar Research Centre, Oxford, UK; 4Clinical and Academic Rheumatology, Kings College Hospital NHS Foundation Trust, London, UK; 4School of Medicine and Biomedical Sciences, University of Sheffield, Sheffield, UK

Background and Objectives: The objective of this study was to define interactions between HLA-DRB1, PTPN22 and smoking in anti-cyclic citrullinated peptide (CCP) and rheumatoid factor (RF) positive and negative rheumatoid arthritis (RA).

Methods: Data from 5020 RA subjects and 3759 healthy controls recruited from six centres across the UK were analysed. The magnitude of association for each genotype and smoking were assessed in autoantibody-positive and negative subgroups. The effect of smoking on antibody status among cases was assessed following adjustment for year of birth using Mantel–Haenszel analysis. Analysis of the joint effects of PTPN22, HLA-DRB1 and smoking were undertaken using additive and multiplicative models of interaction within a logistic regression framework.
Results: The joint effects of PTPN22, HLA-DRB1 and smoking were defined with no evidence of departure from a multiplicative model. Within cases, all three factors were independently associated with the generation of CCP antibodies (odds ratio (OR) 11.1, p<0.0001), whereas only HLA-DRB1 and smoking were independently associated with RF production (OR 4.4, p<0.0001). There was some evidence of an increasing likelihood of antibody positivity with heavier smoking. Finally, we demonstrated that smoking is associated with the generation of both CCP and RF antibodies (OR 1.7, p<0.0001).

Conclusions: PTPN22 appears to be primarily associated with anti-citrulline immunity, whereas HLA-DRB1 remains independently associated with RF in the absence of CCP antibodies. This study has confirmed specific gene–environment combinations that are associated with a substantially increased risk of developing RA. The challenge is to determine how these data can be used to inform clinical practice.

3. B cells and autoantibodies: diagnosis and pathophysiology

A30 AUTOANTIBODIES TO CITRULLINATED PROTEINS/ CITRULLINATED FIBRINogen IMMUNE COMPLEXES INDUCE SECRETION OF TUMOUR NECROSIS FACTOR ALPHA BY MACРОPHAGES FROM RHEUMATOID ARTHRITIS PATIENTS

C Clavel, I Laurent, O Lemaire, L Zabraniecki, B Fourné, M Sebbag, G Serre. Laboratory of Epidermis Differentiation and Rheumatoid Autoimmunity, UMR 5165, CNRS-Université de Toulouse, Toulouse, France; Laboratory of Cell Biology and Cytology, CHU de Toulouse, Toulouse, France; Rheumatology Department, CHU de Toulouse, Toulouse, France

Background and Objectives: Autoantibodies to citrullinated proteins (ACPAs) probably play an important role in rheumatoid arthritis (RA) pathophysiology, because they are produced in the synovial tissue where citrullinated fibrin constitutes their major antigenic target. We developed a human in vitro model in which macrophages, derived from monocytes of healthy controls, were stimulated by immune complexes (IC) comprising ACPA and human citrullinated fibrinogen. It allowed us to demonstrate that the IC induce tumour necrosis factor alpha (TNFα) production by macrophages. We undertook to evaluate the TNFα response they elicit from monocytes and monocyte-derived macrophages of RA patients.

Materials and Methods: Monocytes and monocyte-derived macrophages were obtained from 44 healthy controls and from 26 RA patients diagnosed according to the American College of Rheumatology criteria and undergoing disease-modifying antirheumatic treatment. Fc gamma receptor (FcγR) I, II and III expression was studied by flow cytometry. Cells were stimulated with ACPA-containing IC generated by capturing ACPA from RA sera on immobilised citrullinated fibrinogen. Cell activation was evaluated by TNFα assay in culture supernatants.

Results: Expression of FcγRI and FcγRIII was similar in the monocytes from healthy controls and from RA patients, whereas the proportion of FcγRII-positive monocytes was slightly but significantly lower in RA (91% vs 97% positive cells; p = 0.004). Differentiation of RA monocytes into macrophages was associated with the induction of FcγRIII expression (92% positive macrophages vs 19% positive monocytes; p<0.0001) but with no significant changes in FcγRI expression, as previously observed in healthy controls. The reduction in FcγRI expression, less important than in healthy controls, was not statistically significant. Accordingly, the proportion of FcγRII-positive macrophages was higher in RA (76%) than in healthy controls (52%; p = 0.0007). ACPA-containing IC induced TNFα secretion by monocytes (median 23 pg/ml) and by macrophages (median 68 pg/ml) from RA patients. The ranges of TNFα secretion were roughly similar to those observed with cells from healthy controls. Moreover, in RA macrophages, with only few exceptions, the IC induced secretions of higher amounts of TNFα than in paired monocytes (median ratio ±5; p = 0.0029), as previously observed with cells from healthy controls. Finally, no correlations were observed between TNFα secretion levels and the expression of FcγRI, clinical parameters (disease activity score (DAS28), disease duration) or biological data (erythrocyte sedimentation rate, C-reactive protein, rheumatoid factor and ACPA).

Conclusions: Blood monocytes from RA patients show a decrease in FcγRII expression. The derived macrophages exhibit the same expression of FcγRII and III than macrophages from healthy controls, but an increased expression of FcγRI. The TNFα secretion induced by ACPA-containing IC is five times higher in the RA macrophages than in the paired monocytes. These results reinforce the hypothesis of a direct involvement of ACPA in the pathophysiology of RA.

A31 ANTICITRULLINATED PROTEIN AUTOANTIBODIES HAVE A VERY RESTRICTED DIVERSITY OF RECOGNITION OF CITRULLINATED FIBRIN: THEY TARGET ONLY THREE IMMUNODOMINANT EPITOPES

C Ilobiju, A Magyar, L Noguiera, A Jakab, M Sebbag, F Hudecz, G Serre. Laboratory of Cell Biology and Cytology, CHU de Toulouse, Toulouse, France; ELTE Research Group of Chemistry, Eotvos Lorand University, Budapest, Hungary

Background and Objectives: Among the serological factors associated with rheumatoid arthritis (RA), the most specific are the autoantibodies to citrullinated proteins (ACPAs). Citrullinated fibrin, abundant in the inflamed synovium of RA patients, is a major autoantigen for ACPAs. When mapping the ACPA epitopes on citrullinated fibrin, among 71 15-mer citrullinated peptides derived from the a and b chains, we identified a limited set of 18 peptides bearing such epitopes. Among them, two multicitrullinated peptides, the peptides a56–citr58,42 and b60–74citr60,72 were shown to bear the major epitopes. The purpose of the present study was to analyse the diversity of the ACPA response towards these two immunodominant peptides, then to define the minimal sequential epitopes recognised by ACPA on the peptides.

Material and Methods: Overlapping fibrin heptapeptides encompassing the b60–74 and a36–50 sequences were synthesised as pin-bound uncleavable citrullinated and non-citrullinated, acetylated and non-acetylated peptides. Mono, bi and, when justified, tri-citrullinated forms were synthesised. Three groups of 10 ACPA-positive RA sera were tested by ELISA on these peptides: the a36–50cit38,42-specific sera (a-sera), the b60–74cit60,72-specific sera (b-sera) and the sera recognising both the peptides (ab-sera). A pool of ACPA-negative sera was used as the negative control.

Results: Concerning the reactivity of the b60–74 peptides, as expected, no significant reactivity was observed with the a-sera or the negative control, whereas eight of 10 b-sera and seven of nine ab-sera were reactive towards the same overlapping 7-mer b-derived peptides. Analysing their reactivity towards those peptides allowed a minimal 4/5-mer b epitope, including two citrulline residues, to be defined as target (the “b” epitope). Concerning the reactivity of the a36–50 peptides, as expected, no significant reactivity was observed with the b-sera or the negative control, whereas 10 of 10 a-sera and six of nine ab-sera recognised the same 7-mer overlapping a-derived peptides, allowing a minimal 5-mer a-epitope including the citrulline 42 to be defined as target (the “a” epitope). In addition, two of 10 a-sera and eight of nine ab-sera recognised a third additional overlapping epitope on the a36–50 sequence, including the citrulline 38 (the “a” epitope).

Conclusion: On the two citrullinated fibrin peptides a56–50 and b60–74 previously proved to be the main synovial target of ACPA,
Background and Objectives: Predicting the outcome of rheuma-toid arthritis (RA) is crucial for optimal clinical management. Autoantibodies to citrullinated peptides have proved to be useful prognostic markers. The aim of this study was to test the prognostic capacity of a recently developed test for anti-mutated citrullinated vimentin (MCV) compared with anticyclic citrullinated peptides (CCP) 2 in two independent cohorts of RA patients.

Methods: Results from two independent RA cohorts are included in this study. (1) 82 early RA patients (disease duration <1 year, mean 112 days, 44% rheumatoid factor (RF) positive) were included in this study. (2) The EURIDISS cohort (n = 238, disease duration <4 years, mean 2.3 years, 56% RF positive) was followed longitidually for 10 years. 125 patients with x rays at baseline and follow-up were included in the present analyses. The radiographs were scored according to the van der Heijde modified Sharp score (vdH Sharp score). Both anti-CCP analyses (INOVA) and anti-MCV analyses (ORGENTEC) were performed using ELISA assays according to the manufacturer's instructions. The positive cut-off for anti-MCV was 20 U/l. Patients with an average annual progression in vDh Sharp score of the hands of 1.0 unit or greater were considered progressors (dependent variable). Uni and multivariate logistic regression analyses were performed, as was the Kruskal–Wallis test to compare radiographic progression between groups with varying anti-MCV levels, and linearity checks were performed in the logistic regression.

Results: Both cohorts demonstrated a large overlap in the anti-CCP positive (141/230 and 41/79) and anti-MCV positive patients (148/230 and 45/79). In the EURIDISS/early RA cohort 59/40% showed radiographic progression. In the EURIDISS cohort anti-MCV was a stronger predictor of radiographic progression than anti-CCP (odds ratio (OR) 7.3 (95% CI 3.2 to 16.5) vs OR 3.7 (95% CI 2.6 to 12.5) in univariate analyses. Similar results were found when correcting for disease activity, age, gender and treatment. In the early RA cohort, anti-MCV was also a slightly stronger predictor of radiographic progression than anti-CCP with OR (95% CI) from the multivariate analyses 3.7 (1.2 to 11.7) vs 2.9 (1.0 to 8.5). As previously shown for anti-CCP, a linearity check also established an association between the anti-MCV level and the odds of radiographic progression in both cohorts. Patients with high levels of anti-MCV had a significantly (p<0.01) higher change in the vdH Sharp score than the patients with lower levels in both cohorts.

Conclusion: Despite overlap between anti-CCP and anti-MCV-positive patients, a positive test for anti-MCV was a slightly stronger predictor of radiographic progression than a positive anti-CCP test. This study also indicates that the levels of anti-MCV, in addition to their mere presence or absence, adds to the strength of prediction of radiographic progression.

Background and Objectives: Rituximab is effective in rheumatoid arthritis (RA) but a third of patients fail to achieve a European League Against Rheumatism (EULAR) response at 6 months. Rare event flow cytometry (RE-FACS) showed that blood B-cell depletion is frequently incomplete and that partial depletion predicts poor response. The aim of this study was to find predictors of non-response among demographic, clinical and B-cell depletion parameters as well as synovial tissue analysis. Time of relapse was variable, ranging from 58 to over 104 weeks. We also assessed whether relapse could be predicted from clinical and immunological parameters.

Methods: 59 patients received licensed dose rituximab and had more than 2 years follow-up. Routine clinical and laboratory data were recorded (joint counts, patient-reported outcomes, C-reactive protein (CRP), rheumatoid factor (RF), IgA/M/G). The disease activity score (DAS28) was used to classify response. RE-FACS with B-cell subset analysis (naive, memory and preplasma cells (PFC)) was performed at baseline, after each infusion and then 3 monthly. Biopsies were obtained by arthroscopy. A standard immunohistochemistry technique and digital scoring were used to stain tissue for infiltrating B cells (CD19+) and T cells (CD3+).

Results: 23 were non-responders (33%) and 14 were good responders (24%). Overall baseline DAS28 was 6.77 in responders and 6.17 in non-responders, with a trend towards longer disease duration and lower joint counts in non-responders, but no difference in CRP, erythrocyte sedimentation rate (ESR), HAQ-DI or RF titre. As previously reported, lack of profound depletion (B cells <0.0001 x 10^9/l) predicted non-response (p<0.001). Further subset analyses showed that higher numbers of memory B cells (p = 0.011) and PFC (p = 0.001) at baseline also predicted non-response. Synovial B cells were significantly more numerous in non-responders (p = 0.039, three out of 13 biopsy). We then investigated predictive factors for relapse from parameters at baseline or 6 months in 36 patients initially responding to rituximab. All patients but two relapsed within 2 years. Relapse was therefore divided into early (<1 year post rituximab, n = 19) or late (>1 year, n = 17). A trend was observed for longer relapse-free disease in patients who achieved profound depletion (p = 0.085). B cells returned as soon as week 10, but most frequently at week 26. B-cell return was always preceded by relapse (by 7–78 weeks), but there was no association between B-cell return and early or late relapse. Memory or naive cell numbers were not predictive of relapse; however, memory frequency at 6 months tended to be higher in the early relapse group (median 11% vs 5.5%). Synovial tissue analysis at 6 months did not predict relapse; however, a higher number of T cells at baseline was possibly associated with early relapse (p = 0.065).

Conclusions: At baseline higher circulating memory and PFC numbers as well as more synovial B cells predicts poor response and may indicate a higher burden of disease that may require more intensive therapy such as a higher dose of rituximab. Relapse cannot easily be predicted, possibly because B-cell re-population is highly variable.

Background and Objectives: hsP3-ANCA ELISA is a novel highly-sensitive method for the detection of P3-ANCA in
COMPARATIVE IMMUNOMICS IN HUMANS AND ANIMAL MODELS OF INFLAMMATORY RHEUMATIC DISEASES REVEALS TOLL-LIKE RECEPTOR (7/9) DEPENDENT AND INDEPENDENT AUTOANTIGENS

1K Skrinik, 2Z Konthur, 1T Häupl, 2B Marklein, 1SR Christensen, 1MJ Slomchik, 1A Cape, 1S Steiner, 1H Lehrach, 1GR Burmester, 1Department of Rheumatology and Clinical Immunology, Humboldt University and Free University, Berlin, Germany; 2Max-Planck-Institute for Molecular Genetics, Berlin, Germany; 3Yale University School of Medicine, New Haven, Connecticut, USA; 4Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College, London, UK; 5University Departments of the Vienna Biocenter, Department of Medical Biochemistry, Vienna, Austria

In rheumatoid arthritis (RA) reactions to nucleic acid-binding proteins occur less frequently than in systemic lupus erythematosus (SLE) and its animal models. However, the T-cell-independent activation of certain autoantibody-producing cells such as rheumatoid factor (RF), which receive stimuli via endogenous Toll-like receptors (TLR) may be the initial step to autoimmunity. This study was conducted with sera from rheumatoid arthritis (RA) patients and arthritis animal models to identify common RNA/DNA binding autoantigens and to investigate the role of TLR7/9 for their production.

Methods: Using protein filter technology (30000 human protein–filter, RZPD, Berlin, Germany) the autoantigen profile of RA and SLE patients, mouse collagen and zymosan (SKG)-induced arthritis, as well as collagen and pristane-induced arthritis in rats was obtained. Moreover, sera from TLR7, TLR9-deficient and double-deficient mice with an MRL-lpr/lpr (MRL-lpr) background, as well as collagen and pristane-induced arthritis in rats was obtained. After the intravenous administration of IVIg, the antibody titres to a child with CHB were treated during a subsequent pregnancy with IVIg using a protocol in the Preventive IVIg Therapy for Congenital Heart Block (PITCH Study). All individuals exhibited ELISA reactivity against epitope 349–364 of La/SSB in their initial samples. Sequential sera were drawn from all mothers during pregnancy and evaluated for antibodies against: (1) the epitope 349–364 of La/SSB (before and after the removal of anti-Id antibodies); (2) its complementary epitope (that reacts with anti-Id antibodies); and (3) purified Fab2 fragments of anti-La/SSB autoantibodies.

Results: The evaluation of anti-idiotypic antibodies was successfully performed using either the complementary peptides or purified Fab2 fragments of antibodies against the major epitope 349–364 of La/SSB. Both methods presented a high concordance reaching 90%. After the intravenous administration of IVIg, the antibody titres to the major epitope of La/SSB decreased by 20–60% in four patients and remained stable in one case. Furthermore, there was a substantial increase of anti-id antibody titre in four out of five patients, ranging from 10% to 300%. None of the children developed any conduction abnormality. One child had a rash and one was noted to have fibrosis of the chordae of the mitral valve.

Conclusions: This study demonstrates for the first time that IVIg administration decreases autoantibody titres and enhances the anti-idiotypic antibody response in pregnant women with anti-Ro/SSA and anti-La/SSB antibodies. The beneficial effect of IVIg for the prevention of CHB in high-risk pregnancies awaits studies in larger numbers of patients.
GLYCAN PROFILING OF ANTICITRULLINATED PROTEIN ANTIBODIES

1-3Hil Scherer, 1D van der Waude, 1C Koelman, 2A de Boer, 1T Huizinga, 3A Deelder, 3M Wurthner, 1R Toes. 1Department of Rheumatology, Leiden University Medical Centre, Leiden, The Netherlands; 2Department of Rheumatology and Clinical Immunology, Charité-Universitär Medizin Berlin, Berlin, Germany; 3Department of Parasitology, Leiden University Medical Centre, Leiden, The Netherlands

Background and Objectives: The Fc part of human immunoglobulin G (IgG) carries two N-linked glycan moieties. These glycan moieties are crucial for the structural stability of the antibody and influence its biological activity. In particular, the presence or absence of core fucose residues, galactose residues and sialic acid residues modulate binding affinity to activating or inhibitory Fc receptors and the ability to activate complement. In antibody-mediated autoimmune inflammatory disorders such as rheumatoid arthritis, glycosylation of serum IgG has been shown to be reduced in a disease activity-dependent fashion. Anticitrullinated protein antibodies (ACPA) exhibit unique specificity for rheumatoid arthritis (RA). Factors determining the pathogenicity of ACPA, however, are incompletely understood. We have now developed a technique that allows the analysis of antibody glycosylation in an antigen-specific manner and by such glycan profiling of ACPA.

Methods: ACPA were isolated by affinity purification using citrullinated peptides as antigen. The purity of ACPA isolated by this method was determined by the absence of detectable quantities of IgG in isolates from sera of ACPA-negative RA patients. Following purification, ACPA were subjected to tryptic digest and IgG glycosylation was analysed by Nano-LC-ESI-ion trap mass spectrometry. The glycan profile of ACPA-IgG was compared with the glycan profile of total serum IgG in a set of 85 clinically well-characterised patients.

Results: ACPA were found to exhibit a glycan profile distinct from the profile of total serum IgG. Compared with serum IgG, ACPA lack galactose and sialic acid residues, and show an increased frequency of defucosylated glycoforms. In addition, differences in ACPA glycosylation were detected between rheumatoid factor (RF)-positive as well as RF-negative patients. These subgroup-related differences are specific to ACPA, as they were not detectable in the glycan profile of total serum IgG.

Conclusions: Our data indicate that ACPA IgG exhibit a specific, pro-inflammatory glycan profile, which is distinct from total serum IgG. Moreover, the glycosylation of ACPA, but not of total serum IgG, differs between subgroups of disease. As Fc glycosylation is known to influence biological activity of antibodies these data further our understanding of the mechanisms underlying ACPA pathogenicity.

IMMUNOBLOT ANALYSIS OF ANTI-KU P70 AND/OR P80 ANTIBODIES IN PATIENTS WITH DIFFERENT CONNECTIVE TISSUE DISEASES

4K Lakota, 1A Ceribelli, 1I Cavazzana, 1F Franceschini, 1J Vencovsky, 2B Stamenkovic, 4L Czirjak, 5KM Poljsak, 5SS Semrl, 5B Rozman, 5T Kveder. 1Rheumatology and Clinical Immunology Unit, Spedal Civil, Brescia, Italy; 2Department of Rheumatology, Institute for Prevention and Treatment of Rheumatic and CVD, University of Niš, Niš, Serbia; 3Institute of Rheumatology, Prague, Czech Republic; 4Department of Immunology and Rheumatology, Medical School, University of Pécs, Pécs, Hungary; 5University Medical Centre, Division of Internal Medicine, Department of Rheumatology, Ljubljana, Slovenia

Background: Ku protein is a heterodimer consisting of p70 and p80 subunits involved in DNA repair, transcriptional regulation, telomere maintenance, V(D)J recombination, development of the brain, etc. The prevalence of anti-Ku antibodies was recently evaluated in a European EUSTAR-initiated multicentre case-controlled study with 625 systemic sclerosis (SSc) patients. A clinically distinct subset (2.2%) of these patients was anti-Ku positive and was reported to be associated with myositis, as an important clinical feature. The current study is a continuation of this work.

Aims of the Study: To evaluate the prevalence of anti-p70 and/or anti-p80 antibodies in sera of patients previously determined to be anti-Ku positive by counter-immunoelectrophoresis (CIE).

Materials and Methods: Sera from 70 anti-Ku-positive patients were tested by immunoblotting; out of those 52 had defined vimentin and collagen; furthermore, antibodies specific for cyclic citrullinated filaggrin peptides (CCP) were detected in RA sera and anti-CCP positivity is widely used for diagnostic purposes. The aim of our study was to identify and compare citrullinated epitopes of filaggrin, vimentin and collagen recognised by autoantibodies of RA patients, and to develop a peptide-based diagnostic/prognostic assay suitable for the early diagnosis of RA.

Materials and Methods: We used conventional solid-phase peptide synthesis (Fmoc strategy) carried out on the “MULTIPIN NCP” (Chiron Mimotopes Peptide System) non-cleavable kit. The peptides were prepared in duplicate. Citrullinated peptides and the unmodified counterparts containing arginine instead of citrulline were synthesised on the pins in order to compare their respective reactivities. Pins were covered with approximately 66 nmol peptides. We have used these peptides-on-pins in an “indirect” ELISA and ACPA was determined in the sera samples using anti-IgG plus IgM secondary antibodies in the first experimental series, whereas only IgG-specific antibodies were used in a second set of experiments.

Results: The reactivity of sera from anti-CCP-positive RA patients, anti-CCP-negative non-RA patients and healthy controls with the citrullinated peptides and their arginine-containing counterparts were compared. In the first set of experiments 66 CCP-positive RA, 50 CCP-negative RA and 46 healthy control samples were screened. We identified a 19 mer and two 5 mer citrullinated filaggrin peptides showing a significantly higher reaction with the anti-CCP-positive RA sera as compared with the anti-CCP-negative non-RA and the healthy control samples (p<0.001). When anti-IgG was used as a secondary reagent to detect ACPA, a significant positive correlation was found between the reactivity of the CCP-positive RA sera with the 19 mer and with one of the 5 mer filaggrin peptides and the anti-mutated citrullinated vimentin titre. Both the 5 mer and the 19 mer peptides showed 82.6% selectivity and 65.71% sensitivity for the detection of autoantibodies in the sera of CCP-positive RA patients. We could not detect a significant reactivity with the citrullinated vimentin and collagen peptides.

Conclusion: The identification of these citrullinated filaggrin peptide epitopes could be important for the development of new diagnostic tools for RA.
diagnoses, which were: SSC (14); systemic lupus erythematosus (five); rheumatoid arthritis (five); systemic myositis (three); undifferentiated connective tissue disease (19) and overlapping syndrome (six). 26 blood donors were also tested. Before Western blotting, nuclear lysates from THP-1 cells were immunoprecipitated with protein A/G plus agarose using anti-Ku mouse monoclonal antibody 5 (clone 162) targeted against the native Ku heterodimer. The immunoprecipitate was transferred onto 10% sodium dodecylsulphate–polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane and cut into strips. Patient sera were incubated at a dilution of 1 : 50, followed by secondary goat antihuman IgG-horseradish peroxidase (HRP) at a dilution of 1 : 1000. Detection of p70/p80 subunits of Ku was performed with Western blotting luminol reagent and chemiluminescence was detected with G:Box. As positive controls goat polyclonal anti-Ku-86 and anti-Ku-70 were detected with secondary donkey anti-goat IgG-HRP.

Results: 58% of all patients were positive for both anti-p70 and anti-p80. Only 10% of patients who were CIE anti-Ku positive, were negative for both subsets of antibodies in the immunoblot— with an apparent conformational epitope of the antigen. 27% of patients were positive for only anti-p70, whereas 6% of patients had only anti-p80 antibodies present. No association with specific diagnoses was found in either group.

Conclusion: 90% of all Ku-positive (CIE) patients have been confirmed using Western blotting. Overall, there is a 20% higher prevalence of the p70 subunit compared with p80. However, the number of patients in the subgroups is limited and in the future, clinical association will be further sought.

[**A40**] MARKED DIFFERENCES IN FINE SPECIFICITY AND ISOTYPE USAGE OF THE ANTICITRULLINATED PEPTIDE RESPONSE IN HEALTH AND DISEASE

1 A lean, ‘A Willemze, ‘D Robinson, ‘C Peschken, ‘J Markland, ‘D van der Woude, ‘B Elias, ‘HA Ménard, ‘M Newkirk, ‘M Frizier, ‘RE Toes, ‘TWJ Huizinga, ‘H El-Gabalawy. 1Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands; 2Arthritis Centre, University of Manitoba, Winnipeg, Manitoba, Canada; 3University of Saskatchewan, Saskatoon, Saskatchewan, Canada; 4Division of Rheumatology, McGill University Health Center, Montreal, Quebec, Canada; 5University of Calgary, Calgary, Alberta, Canada

Background and Objectives: Anticitrullinated peptide antibodies (ACPA) display a high association with rheumatoid arthritis (RA) and have been implicated in its pathogenesis. ACPA are also known to precede RA onset and predict disease development. Therefore, we have aimed to identify the features of the ACPA response that could confer its pathogenicity by extensively characterising this antibody response in a unique north American native population of RA patients and their unaffected relatives.

Materials and Methods: IgA, IgM and IgG ACPA were measured in serum from 81 RA patients and 195 unaffected relatives, using cyclic citrullinated peptide 2 plates (Euro-Diagnostica, Arnhem, The Netherlands). Likewise, IgA and IgM rheumatoid factor (RF) were measured. The isotype distribution, fine specificity of the ACPA response and its association with RF were compared in health and disease.

Results: Approximately 20% of the healthy relatives and 90% of RA patients were positive for ACPA. ACPA isotype usage was strikingly lower in unaffected relatives (one to two isotypes) than in RA patients (five to six isotypes). Fine specificity studies showed that reactivity to citrullinated fibrinogen and vimentin was present in the RA sera, whereas 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, whereas the association of RF with disease relied on its interaction with ACPA.

Conclusion: 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 the development of RA and this process could be facilitated by the presence of RF antibodies.

[**A41**] NEW AUTOANTIGENS IN RHEUMATOID ARTHRITIS BY SCREENING PROTEIN ARRAYS

1 I Auger, 2’N Balandraud, 3’J Raik, ‘N Lambert, ‘M Martin, 1’J Roudier. 1INSERM UMR 629, Marseille, France; 2APHM, La Conception, Marseille, France

Objectives: Two elements are central to the development of rheumatoid arthritis (RA): HLA-DR alleles and autoantibodies. The risk of developing RA for an individual is controlled by the two HLA-DR alleles he expresses. HLA-DR alleles can be susceptible, protective or neutral towards the development of RA. Two susceptible alleles can cooperate and contribute a very high risk to develop RA. The most critical autoantibodies in RA are directed at citrullin residues on proteins such as fibrin, filaggrin and vimentin. Non-citrullinated proteins can also be the target of autoantibodies in RA. To identify new autoantibodies in RA, we selected sera from RA patients to screen protein arrays.

Patients and Methods: Serum from 19 RA patients with given HLA-DR genotypes, from seven spondylarthropathy patients, two lupus patients, four systemic sclerosis patients and 10 healthy individuals were used to probe arrays containing 8268 human proteins. All proteins had been expressed as glutathione-S-transferase fusion proteins, purified under native conditions, and spotted in duplicate on nitrocellulose array. Serum samples were added to the arrays. The presence of autoantibodies bound to each protein was detected by a fluorescently labelled anti-human IgG antibody.

Results: On average, patients’ and controls’ sera recognise 100 of the 8268 human proteins expressed on the array. Most of the proteins recognised by RA sera are also recognised by control sera. There was no difference in the number of recognised proteins between RA patients expressing two, one or no RA-associated HLA-DR alleles. Four proteins were recognised almost uniquely by sera from RA patients. These proteins are peptidyl arginine deiminase 4 (PAD4), protein kinase C beta 1 (PKCβ1), phosphatidylinositol 4 phosphate 5 kinase type II gamma (PIP4K2C) and vraf murine sarcoma viral oncogene homolog B1 (BRAF). Using purified proteins, we confirmed that PAD4 and BRAF are recognised almost uniquely by RA patients.

Conclusions: We found and validated two major autoantigens recognised by RA patients: BRAF and PAD4.

[**A42**] PROGNOSTIC ASSOCIATIONS OF IGG ANTIBODIES AGAINST CITRULLINATED ALPHA-ENOLASE PEPTIDE 1 IN EARLY RHEUMATOID ARTHRITIS


Background and Objective: Patients with rheumatoid arthritis (RA) who are positive for anticyclic citrullinated peptide (CCP) antibodies have poorer long-term prognosis compared with anti-CCP-negative patients. The anti-CCP-positive RA patients also demonstrate an association with HLA-DRB1 shared epitope alleles and cigarette smoking earlier found in anti-CCP-unselected sera. This gene/environmental association has recently been shown to be almost totally confined to the citrullinated alpha-enolase peptide 1 (CEP-1)-positive subgroup of anti-CCP-positive RA patients, arguing for citrullinated alpha-enolase as a critical autoantigen in RA. Here we have investigated the prognostic impact of anti-CEP-1 in early RA.

Conclusion: 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 the development of RA and this process could be facilitated by the presence of RF antibodies.
Methods: 272 early RA patients with 5-year follow-up data (C-reactive protein, erythrocyte sedimentation rate, pain visual analogue scale, global visual analogue scale, health assessment questionnaire, physician’s assessment, swollen joint count, tender joint count, disease activity score (DAS28), 2 first years Larsen scores), earlier investigated concerning anti-CCP (Rønnested et al, 2005), were assayed by ELISA, using the CEP-1 peptide and the arginine-containing control peptide as coating antigens. The cut-off was defined as the 95th percentile of 100 healthy blood donors. The prognosis was analysed by comparing anti-CEP-1-positive and negative patients at different time points after diagnosis in non-parametric design, either in the total RA cohort or within the anti-CCP-positive subgroup. Anti-CEP-1-positive patients with higher optical density values in the control wells (n = 6) were excluded from the analysis.

Results: 150/266 (56%) patients were anti-CCP positive, whereas 68 (26%) were anti-CEP-1 positive at baseline. 62/68 anti-CEP-1-positive patients were also anti-CCP positive. In the total cohort, anti-CEP-1 was associated with more swollen joints and higher disease activity, as estimated by a rheumatologist after 3 and 5 years, as well as with a marginally higher erosion rate between 1 and 2 years. This difference was much smaller than the earlier published difference between anti-CCP-positive and negative patients. Within the anti-CCP-positive subgroup, anti-CEP-1-positive and negative patients did not differ concerning any measure at any time point.

Conclusions: In this study, baseline anti-CEP-1 does not predict any differences in future disease activity and severity, compared with baseline anti-CCP. The differences between anti-CEP-1-positive and negative patients in the total cohort are probably secondary to the close anti-CEP-1/anti-CCP association. Although anti-CEP-1-positive patients are a distinctive subset within the anti-CCP-positive group in terms of environmental and genetic risk factors, the downstream events, as clinical manifestations, appear to be similar.

A44 SERUM LEVELS OF B-CELL ACTIVATING FACTOR CORRELATE WITH ANTI-JO-1 AUTOANTIBODY LEVELS IN PATIENTS WITH INFLAMMATORY MYOPATHIES

1O Kryšťufková, 1M Modrá, 1H Mann, 1Půtová, 1E Lundberg, 1J Vencovský.
1Immunology and Clinical Department, Institute of Rheumatology, Prague, Czech Republic; 2Rheumatology Unit, Department of Medicine, Karolinska University Hospital, Solna, Stockholm, Sweden

Background: Antihistidyl-tRNA synthetase (anti-Jo-1) antibody is a myositis-specific autoantibody. A correlation of anti-Jo-1 autoantibody serum levels with disease activity in patients with myositis has been reported. B-cell activating factor (BAFF) of the tumour necrosis factor family BlyS is crucial for B-cell maturation and survival and plays a role in autoantibody production. We have recently reported elevated serum levels of BAFF in patients with dermatomyositis and patients with polymyositis and anti-Jo-1 autoantibodies. A correlation between levels of BAFF and serum creatine kinase (CK) was also present. The aim of this study was to investigate whether serum BAFF correlates with anti-Jo-1 antibody levels in patients with myositis and to find out if they are related to laboratory markers of muscle disease and to inflammatory activity.

Materials and Methods: BAFF and anti-Jo-1 levels were measured by ELISA (RnD and Oregene) in 69 serum samples from 21 patients with dermatomyositis and 28 with polymyositis. Longitudinal paired samples from 11 dermatomyositis and 10 polymyositis patients were also evaluated. The anti Jo-1 positivity was initially detected by line-blot and Western blot assays. Levels of CK, myoglobin, lactate dehydrogenase aminotransferases (alanine aminotransferase, aspartate aminotransferase) and C-reactive protein (CRP) in sera were retrieved from patients’ records or measured in the same sera.

Results: A significant correlation was found between levels of BAFF and anti-Jo-1 antibodies in sera (rs = 0.42; p = 0.0003). Both BAFF and anti-Jo-1 serum levels correlated with CK (rs = 0.4; p = 0.0008 and rs = 0.59; p = 0.0012), myoglobin (rs = 0.28; p = 0.025 and rs = 0.29; p = 0.021) and CRP (rs = 0.47; p<0.0001 and rs = 0.4; p = 0.001). Serum levels of muscle enzymes and myoglobin correlated with each other, but no correlation was found with CRP. The changes in BAFF and anti-Jo-1 in longitudinal paired samples correlated positively (rs = 0.44; p = 0.0425), but after the exclusion of two outliers the correlation became borderline (rs = 0.42; p = 0.065).

Conclusion: The correlation of serum BAFF and anti-Jo-1 levels and their association with markers of muscle involvement suggest that both proteins are related to pathways implicated in the disease pathogenesis and perhaps mediating processes leading to active illness. BAFF may be responsible for the production of anti-Jo-1 autoantibodies, or anti-Jo-1 could cause elevation of serum BAFF by its type I interferon inducing capacity. There could be an additional non-specific inflammatory mechanism involved in the production of both BAFF and anti-Jo-1 autoantibodies, which is suggested by the simultaneous elevation of serum CRP.

Finance: This study was supported by AutoCure LS HB CT-2006-018661 funding and institutional support MSM 0021620812 from the Ministry of Education, Youth and Sports in the Czech Republic.
A45 SYNVOIAL TISSUE SUBLINING CD68 EXPRESSION AS A BIOMARKER OF THERAPEUTIC RESPONSE IN RHEUMATOID ARTHRITIS CLINICAL TRIALS: CONSISTENCY ACROSS CENTRES

Background and Objectives: From studies at one academic centre (Amsterdam Medical Center; AMC), CD68 expression in synovial tissue has been proposed as a biomarker of therapeutic response in randomised clinical trials. The aim of this study was to determine whether the correlation between the mean change in disease activity and the mean change in synovial sublining CD68 expression could be demonstrated across different academic centres.

Material and Methods: Synovial biopsies obtained from patients with rheumatoid arthritis before and 16 weeks after rituximab therapy were selected and coded. Paired sections were stained for CD68 independently at AMC and at St Vincent’s University Hospital (SVUH), Dublin. Digital image analysis was employed at both centres to quantify CD68sl expression. After decoding, inter and intracentre variations in quantification of CD68sl expression, ∆CD68sl, and the relationships between ∆CD68sl and ∆DAS were determined. Determination of the sensitivity of biomarker expression to detect change was based on the standardised response mean (SRM).

Results: Initial weak agreement between centres in ∆CD68sl expression was explained by methodological issues, which were resolved. After this learning phase, high levels of intracentre and intercentre agreement were observed. For the pooled sections stained at AMC, the correlation between two investigators was R = 0.942, p = 0.000, and for sections stained at SVUH R = 0.899, p = 0.001. Similarly, the intracentre correlations for ∆CD68sl expression after treatment were R = 0.998, p = 0.000, for sections stained at AMC and R = 0.880, p = 0.000, for sections stained at SVUH. The intercentre correlation for the pooled scores of sections stained at AMC was R = 0.85, p = 0.000 and for the sections stained at SVUH R = 0.62, p = 0.001. The consistent correlation between ∆DAS and ∆CD68sl expression across different studies (Pearson correlation 0.895, p<0.001) was confirmed. The SRM values for ∆CD68sl, calculated from analyses at both AMC and SVUH were consistently 0.5 or greater, indicating a moderate to high potential to detect change.

Conclusions: The correlation between mean ∆DAS and mean ∆CD68sl expression was confirmed across two centres. Examination of serial biopsy samples can be used reliably to screen for interesting biological effects at the site of inflammation at an early stage of drug development.

A46 THE ANTICYCLIC CITRULLINATED PEPTIDE 2 ISOTYPE REPertoire REFLECTS RHEUMATOID ARTHRITIS DISEASE PROGRESSION AND SEVERITY

1D van der Woude, 2S Syversen, 3K Verpoort, 4E van der Voort, 2G Goll, 1M van der Linden, 11A van der Helm-van Mil, 1D van der Heijde, 1T Huizinga, 3K Veen, 2R Toes.

1Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands; 2Department of Rheumatology, Diakonhjemmet Hospital, Oslo, Norway

The first two authors contributed equally.

Objective: Antibodies against cyclic citrullinated peptides (CCP) are the most powerful predictive factor for the development and progression of rheumatoid arthritis (RA). The anti-CCP response has been shown to consist of various isotypes, but the implications of differences in isotype repertoire have not been extensively investigated. In this study, we used data from two independent cohorts of arthritis patients to investigate the relationship between anti-CCP2 isotypes and disease progression and severity.

Methods: Anti-CCP2 isotypes were determined by ELISA in the sera of anti-CCP2-positive patients. To examine disease progression, the isotype distribution in baseline sera of patients with undifferentiated arthritis (UA) who did not have disease progression (UA-UA), was compared with the isotype profile of UA patients who developed RA (UA-RA). To investigate disease severity, long-term radiographic follow-up data (Sharp–van der Heijde scores) were compared among anti-CCP2-isotype-positive and negative patients in two independent cohorts.

Results: The number of anti-CCP2 isotypes within anti-CCP2-positive patients was a significant predictor of disease progression from UA to RA, with more isotypes indicating a higher risk of RA development (odds ratio for every additional isotype 1.7, 95% CI 1.3 to 2.2, p<0.001). Anti-CCP2 isotypes supplied additional prognostic information to CCP2 status alone, even after correction for other predictive factors. In both cohorts, radiographic damage could also be predicted by the number of anti-CCP2 isotypes and especially by the presence of IgA and IgG3-anti-CCP2.

Conclusion: The anti-CCP2 isotype repertoire reflects disease progression from UA to RA and disease severity. These results indicate that not only the presence, but also the exact constitution of the anticyclulin immune response, is relevant for the disease course of RA.

A47 THE FUNCTIONAL ROLE OF IGE-ANTICITRULLINATED PEPTIDE/PROTEIN ANTIBODIES IN RHEUMATOID ARTHRITIS

AJM Schuwerweg, A Ioan, AD Jorjine, EH van der Voort, TWJ Huizinga, REM Toes. Leiden University Medical Center (LUMC), Department of Rheumatology, Leiden, The Netherlands

Background: Rheumatoid arthritis (RA) is a systemic autoimmune disease. Among the autoantibodies described in RA, anticitrullinated peptide/protein antibodies (ACPA) are highly specific and predictive for RA. Different isotypes of ACPA have been described in RA patients. Because mast cells have recently been shown to play a crucial role in the induction of arthritis in mice, we hypothesised that citrullinated proteins activate mast cells by cross-linking specific IgE-ACPA molecules. Thereby, mast cells and other FcεRI-positive cells could act as important effector cells in RA. The objective of this study is to investigate the presence and the functional role of IgE-ACPA in RA patients.

Materials and Methods: Anticitrullinated protein (fibrinogen) IgE antibodies were assessed with a sandwich ELISA. The potency of these sera to activate basophils via the human FcεRI was evaluated by performing a passive sensitisation of human FcεRI transfected rat basophil cells (RBL). To assess the ability of citrullinated proteins to activate basophils directly, a basophil activation test was performed with peripheral blood of ACPA-negative and ACPA-positive RA patients and healthy controls. Visualisation of citrullinated protein binding to basophils was performed by
FACs. Synovium of RA patients and osteoarthritis patients was obtained by joint replacement surgery or arthroscopic biopsy. IgE and FcεRI expression was investigated by FACs on synovial mast cells. Histamine (competitive ELISA) and IgE levels (UniCap) were measured in the synovial fluid of ACPA-negative, ACPA-positive RA and osteoarthritis patients.

**Results:** IgE antibodies against citrullinated antigens, but not against their uncitrullinated counterparts, could be detected in the sera of ACPA-positive RA patients. Furthermore, serum from ACPA-positive RA patients could specifically sensitise human FcεRI-expressing RBL cells. Non-transfected RBL cells were not activated, showing the ability of citrullinated antigens to crosslink IgE-ACPAs via FcεRI. Direct ex-vivo activation (ie, within 20 minutes) of basophils after exposure to citrullinated antigens (but not uncitrullinated control antigens) was observed in ACPA-positive RA patients in contrast with ACPA-negative RA patients and healthy controls. This was strictly correlated with the binding of citrullinated proteins in basophils and the presence of immunoglobulins on the surface of these cells. In synovial mast cells, FcεRI and IgE expression was increased in ACPA-positive patients compared with ACPA-negative patients and osteoarthritis patients. Histamine levels correlated with IgE levels in ACPA-positive RA synovial fluid, but not in ACPA-negative RA, suggesting degranulation of mast cells by crosslinking IgE-ACPAs.

**Conclusions:** Our data show for the first time a direct biological response to citrullinated antigens of immune cells in ACPA-positive RA patients only. Furthermore, our data indicate that IgE-ACPAs and FcεRI-positive cells (such as mast cells) are involved in the pathogenesis of RA.

**A48 PROTEIN CITRULLINATION BY PORPHYROMonas GINGIVALIS: A PROMISING PIECE IN THE CITRULLINE PUZZLE**

1N Wegner, 2S Eick, 3A Stoka, 4K-A Nguyen, 5J Potempa, 1P Venables. 1Kennedy Institute of Rheumatology, Imperial College London, London, UK; 2Department of Medical Microbiology, University Hospital of Jena, Jena, Germany; 3Department of Microbiology, Jagiellonian University, Krakow, Poland; 4Institute of Dental Research, Westmead Centre for Oral Health, Sydney, Australia; 5Department of Biochemistry, University of Georgia, Athens, Georgia, USA

**Background and Objectives:** The presence of autoantibodies to citrullinated proteins is a specific feature of rheumatoid arthritis (RA). It is unknown which factors initiate the anticitrullinated protein antibody response, which can precede clinically apparent RA by several years. Chronic periodontal infection with *Porphyromonas gingivalis* is a possible risk factor, as this major periodontopathogenic bacterium was previously reported to express a peptidylarginine deiminase (PAD)-like enzyme that could generate citrullinated antigens in a “danger signal” context. It is unclear, however, whether and to what extent *P. gingivalis* citrullinates its own proteins in vivo, and whether this is a specific feature of *P. gingivalis* or is common among oral bacteria. Here, we investigated the presence of endogenously citrullinated proteins in *P. gingivalis* and 13 other oral pathogens and commensals, as a possible mechanism for the induction of anticitrulline autoimmunity in RA.

**Materials and Methods:** Bacterial strains were grown to the stationary phase and lysed by sonication. Gingival crevicular fluid was collected from diseased periodontal pockets in patients with severe periodontitis. Soluble cell lysate and crevicular fluid proteins were resolved by sodium dodecylsulphate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes for the detection of citrullinated proteins using the anti-modified citrulline antibody. For PAD gene analysis, genomic DNA was isolated using standard protocols and subjected to PCR using primers for amplification of the published full-length *P. gingivalis* PAD gene.

**Results:** A number of endogenously citrullinated proteins could be found in all tested *P. gingivalis* strains (two reference strains and five clinical isolates). None of the 15 control oral pathogens and commensals, including the closely related but less periodontopathogenic bacterium *Porphyromonas endodontalis*, expressed citrullinated proteins. The presence of citrullinated proteins correlated with that of the bacterial PAD gene, as confirmed by PCR. Citrullination was also demonstrated in gingival crevicular fluid, an inflammatory exudate from the gingival crevice.

**Conclusions:** This study demonstrates that protein citrullination occurs not only in mammalian cells but also in bacteria. Among those bacteria analysed, this is unique to *P. gingivalis*, confirming the presence of a functional bacterial PAD enzyme. We also showed citrullination in vivo at the site of gingival inflammation, which could originate both from *P. gingivalis* and human cells. These results suggest that citrullinated proteins from *P. gingivalis* are a candidate source for priming the anticitrullinated protein antibody response, which characterises RA.

**A49 IGA RHEUMATOID FACTOR IS AN ADDITIONAL RISK FACTOR FOR EXTRA-ARTICULAR DISEASE (NODULES) IN RHEUMATOID ARTHRITIS**

1 Dr Patel, 2H Pouleom, 3D Plant, 4PJ Charles. 1Division of Immunology Hammersmith Hospitals NHS Trust, London, UK; 2ARC Epidemiology Unit, University of Manchester, Manchester, UK; 3Kennedy Institute of Rheumatology, Faculty of Medicine, Imperial College, London, UK

**Background:** Recent years have seen the emergence and establishment of antibodies to citrullinated antigens as a significant diagnostic marker for rheumatoid arthritis (RA). Although these antibodies are more specific for the diagnosis of RA than rheumatoid factors (RF), there is increasing evidence that RF still have a role to play as serological markers of disease severity or therapeutic response. In this study we have examined the frequency of IgA RF directed at human and rabbit Fc gamma to establish their frequency and sensitivity in RA, together with their genetic and clinical associations.

**Methods:** We have examined a well-characterised RA cohort (the National Repository), collected by the ARC Epidemiology Unit, University of Manchester. Patients were recruited from hospital specialist units within the UK. IgA RF was measured by an addressable bead laser immunoassay (BMD, France). IgM RF was measured using passive particle agglutination (Mast Diagnostics, UK).

**Results:** IgA RF was detected in 136/328 (41.4%) patients with RA. There was no observed correlation between the presence of IgA RF and anticyclic citrullinated peptide (CCP) 2 antibodies or between the levels of the two autoantibodies (r = 0.27). There was no observed association between the presence of IgA RF and the expression or copy number of 01 alleles. IgA RF was associated with the shared epitope containing HLA-DR alleles (odds ratio (OR) 2.83, 95% CI 1.27 to 6.01, p = 0.01) but not erosions. IgM RF was associated with nodules (OR 2.55, 95% CI 1.22 to 5.31, p = 0.012) and erosions (OR 1.98, 95% CI 1.00 to 3.93, p = 0.05). As a result of the co-existence of IgA RF with IgM RF in the majority of sera, additional analyses were performed to examine the independent risk conferred for nodules by IgA RF. At an interpretation threshold of 1:40 for IgM RF and 40 μg/ml for IgA RF, this showed a significant association (p = 0.05) for an association with IgA-positive/IgM-positive RF over and above that observed for IgM RF positive alone.

**Discussion:** IgA RF was detected in 41.4% of patients with RA. The use of both potential target antigens increased the detection rate over the use of a single Fc gamma species. However, there were no observed differences between the associations of the two individual species. There was no association with the presence of shared epitope alleles or of shared epitope allele copy numbers. There was also no association observed between anti-CCP2 and IgA RF. The presence of both IgA and IgM RF was associated with an increased risk of the development of nodules. The risk associated with IgA RF was additional to the risk conferred by the presence of
IgM RF. IgA RF is a candidate risk factor for the development of nodules and potentially other extra-articular manifestations in patients with RA, and it may provide a useful analytical tool in the subclassification of patients with RA into high and low-risk groupings for therapeutic stratification.

4. T cells in autoimmunity

A50 CONSTITUTIVE EXPRESSION OF THE CO-STIMULATORY MOLECULE CD70 BY NON-ANTIGEN-SPECIFIC B CELLS IS SUFFICIENT TO DRIVE T-CELL-DRIVEN AUTOIMMUNE DISEASE

1GF Salinas, 1T Cantaert, 1M Noote, 1E Betelli, 1V Kuchroo, 1P Pap, 1R van Lier, 1D Baeten. 1Department of Clinical Immunology and Rheumatology, Academic Medical Center, Amsterdam, The Netherlands; 2Department of Experimental Immunology, Academic Medical Center, Amsterdam, The Netherlands; 3Center for Neurological Diseases, Brigham and Women’s Hospital, Harvard Medical School, Boston, USA

Objective: B cells are critical for experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG) protein but not by MOG peptides. Therefore, double transgenic mice with B and T cells specific for MOG showed a higher incidence and severity of spontaneous disease and have a different phenotype than mice with MOG-specific T cells only. In this model, B cells contribute to disease by T-cell activation rather than by autoantibody production but it remains to be established whether their main role is to present antigen or to provide costimulatory signals to the T cells. To address this question we studied the development of EAE induced by MOG-specific T cells in the presence of non-antigen-specific B cells that constitutively express the co-stimulatory molecule CD70.

Materials and Methods: Double transgenic mice were bred by crossing 2D2 mice (in which 95% of T cells are specific for the immunodominant MOG peptide) with CD70+ mice (with transgenic expression of the co-stimulatory molecule CD70 under the CD19 promoter, leading to constitutive expression by B cells). 2D2+CD70+ animals were compared with their 2D2-CD70−, 2D2−CD70+, and wild-type littermates. Mice were scored for spontaneous EAE symptoms on a four-point scale over a 20-week period.

Results: During the experiment, none of the animals in the three control groups developed any signs of neurological disease. On the contrary, five of 15 (33%) 2D2+CD70+ mice developed spontaneous EAE. The mean maximal clinical score was 2.8 and the mean age of onset 73 days. Phenotypic analysis of the spleen lymphocytes revealed an increase in CD4 T lymphocytes, with a relative decrease in CD44 high, CD62 high, naive T cells in the 2D2+CD70+ mice versus the 2D2+ animals, indicating that more MOG-specific T cells get primed. Moreover, there was a significant increase in IFNγ production by CD4 T cells in the 2D2+CD70+ mice upon non-specific stimulation in vitro. The number of Foxp3+ regulatory cells was also clearly reduced in these double transgenic animals.

Conclusions: The constitutive expression of the co-stimulatory molecule CD70 by non-antigen-specific B cells is sufficient to lead to T-cell-driven autoimmune disease in this model. The functional profile of the pathogenic T cells and the corresponding immunopathology are currently under investigation.

A52 ELEVATED LEVELS OF IL-15 IN RHEUMATOID ARTHRITIS BONE MARROW CONTRIBUTE TO T-CELL ACTIVATION AND IL-17 OVERPRODUCTION

1E Warnawin, 1A Radzikowska, 1T Burakowski, 1W Rudnicka, 1P Maldyk, 1W Maslinski. 1Department of Pathophysiology and Immunology, Institute of Rheumatology, Warsaw, Poland; 2Department of Rheumorthopaedic Surgery, Institute of Rheumatology, Warsaw, Poland

Background: Recent data indicate that bone marrow plays a role not only as a primary lymphoid organ responsible for haematopoiesis, but also as a secondary lymphoid organ with the capability of antigen presentation exceeding that of lymph nodes. Although in chronic inflammatory/immune disease, such as rheumatoid arthritis (RA), bone marrow participates in the initiation and/or perpetuation of the disease, there is little information about the real number of lymphocyte subpopulations in the bone marrow of these patients and how their function can be modulated by the T-cell growth factor, IL-15.

A51 DESPITE INCREASED NUMBERS OF IL-7 RECEPTOR–FOXP3–EXPRESSING T CELLS, IL-7-INDUCED IMMUNE ACTIVATION IS NOT PREVENTED IN PATIENTS WITH PRIMARY SJÖGREN’S SYNDROME

A Bikker, FM Monet, JWJ Bijlsma, AA Kruize, FPJG Lafeeper, JAG van Roon. Rheumatology and Clinical Immunology, UMC Utrecht, Utrecht, The Netherlands

Background: Evidence is accumulating that IL-7 contributes to inflammation in primary Sjögren’s syndrome (pSS). IL-7 is abundantly expressed in glandular tissue of patients with pSS and activates T cells in these patients. Recently, a specific subset of T cells, expressing the IL-7 receptor (IL-7R) was classified as a pro-inflammatory responder T cell. These T cells could potentially activate B cells and monocytes and also induce tissue damage. In contrast, T cells lacking IL-7R and that express CD25 have a highly suppressive function. A shift in balance between these two opposing subsets could lead to an increase in local inflammation and eventually tissue destruction. The purpose of our study was to examine the balance between IL-7R+ responder T cells and IL-7R– regulatory T cells (Treg) in pSS patients versus healthy controls and the functional consequences this has.

Methods: Expression of CD25, CD127 (IL-7R) and FoxP3 on CD4 T cells from 13 pSS patients and 11 healthy controls was assessed using FACS analysis. To determine the distribution of CD25, CD127 and FoxP3 within naive and memory T cells, isolated CD4 T cells from six patients and six healthy controls were stained with CD45RO and CD27. To test the functional properties of the T-cell subsets in vitro, they were co-cultured with monocytes and the effect of IL-7 and several (super)antigens on proliferation of CD4 T cells from six pSS patients was measured and compared with healthy controls.

Results: In contrast to CD25+ T cells, CD127+ and CD25+CD127− T cells were significantly increased in pSS patients compared with healthy controls (19.4% vs 16.5%, p = 0.02; 6.9% vs 2.4%, p = 0.001; 2.7% vs 1.4%, p = 0.02, respectively). Subsets of CD25+, CD127− and CD25+CD127+ T cells contained a high percentage of Foxp3-expressing T cells (40.6%, 56.1% and 93.7%, respectively). No significant difference was found for FoxP3 expression in these subsets between patients and healthy controls. Total CD4 T-cell FoxP3 expression was significantly increased in pSS patients compared with healthy controls (4.9 vs 8.4, p = 0.08), mainly due to slight increases in FoxP3 expression in CD25− (p = 0.02) and CD127+ T cells (p = 0.02). The naive CD4+CD27+CD45R− T-cell pool hardly contains any CD25+CD127− Treg, but an increase in Foxp3-expressing CD25+CD127− Treg is observed with differentiation from memory towards effector cells. The percentage of FoxP3+ expressing cells in the memory CD25+CD127− T-cell subset of pSS patients was similar to that of healthy controls (91% vs 92%, NS). Increased CD127-Treg numbers in pSS patients are associated with decreased superantigen-induced T-cell proliferation (p<0.05), but not with significant changes in IL-7-induced proliferation in these patients compared with healthy controls.

Conclusions: This study reveals a T-cell imbalance in pSS patients with an increase in CD25+CD127− Treg. Our data indicate that although this could result in the suppression of superantigen-induced T-cell activation, IL-7 can cause persistent T-cell-driven immune activation in pSS.
**Objectives:** (1) To compare the real T-cell numbers in bone marrow isolated from RA and osteoarthritis patients; (2) To measure the levels of soluble IL-15, IL-17, IL-23 and transforming growth factor beta (TGFβ) in bone marrow plasma; (3) To analyse the expression of IL-15Rα on T cells in bone marrow; (4) To analyse the influence of IL-15 stimulation on IL-17 production; (5) To analyse the influence of IL-15 stimulation on T-cell proliferation.

**Materials and Methods:** Bone marrow samples were obtained from patients undergoing joint replacement surgery. Levels of IL-15, IL-17, IL-23 and TGFβ were measured using specific ELISA. The real number of lymphocytes stained for CD3+, CD4, CD8 were counted in the presence of TruCount beads using flow cytometry. IL-17 production and T-cell proliferation (measured as a Ki-67 expression) were evaluated by flow cytometry.

**Results:** Bone marrow from RA patients contained double CD3+ T cells in comparison with osteoarthritis (6.1 ± 2.8 vs 3.2 ± 1.6 × 10⁶ cells/ml, p = 0.003). The ratio CD3+CD4+/CD3+CD8+ was increased in RA bone marrow, clearly indicating the accumulation of CD4 cells. Both CD3+CD4+ and CD3+CD8+ cells proliferated after IL-15 stimulation in vitro. Differences between the osteoarthritis and RA group in proliferation after IL-15 stimulation was observed only for CD3+CD4+ cells. There were elevated levels of IL-15 (1004.5 ± 956.3 pg/ml and 760 ± 238.7 pg/ml, respectively, p = 0.01) and IL-17 (582.4 ± 414.5 pg/ml and 285 ± 223.67 pg/ml, respectively, p<0.05) in bone marrow plasma from RA in comparison with osteoarthritis patients. Similar levels of IL-23 and TGFβ in RA and osteoarthritis bone marrow suggest that these cytokines do not play a significant role in observed differences including Th17 cells. In contrast, elevated levels of IL-15 suggested its participation in IL-17 induction. Indeed, RA bone marrow mononuclear cells produced higher levels of IL-17 after in-vitro stimulation by IL-15 than osteoarthritis (measured by ELISA, p<0.05; or flow cytometry, p<0.04).

**Conclusion:** Our results suggest that locally overproduced IL-15 is responsible for the activation and proliferation of T cells in situ, reflected by significantly increased numbers of activated T cells in RA bone marrow. The accumulation and activation of CD3+CD4+ cells leads to IL-17 overproduction in situ that may actively contribute to the pathogenesis of RA.

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kappa reporter plasmid containing Jurkat cells were used to study the role of NO in nuclear factor kappa B (NF-kB) activation.

**Results:** Our present data indicate that TNF 5–50 ng/ml down-regulates CD3 gamma delta chain in Jurkat cells, by contrast total and cell surface CD3 epsilon protein expression is not downregulated by TNF. NO donor NOS-18 inhibits both TNF and CD3/CD28 stimulation-induced NF-kB activation (p<0.001 and p<0.001, respectively) in a dose-dependent manner. NOS-18 pretreatment inhibits TNF-induced CD3 gamma delta downregulation, by contrast, NF-kB inhibitor peptide treatment does not alter TNF-induced CD3 gamma delta downregulation.

**Conclusions:** These data suggest that TNF regulates T-cell responsiveness by regulating CD3 gamma delta expression in a NO-dependent way, thereafter an increased TNF level may contribute to the hyporesponsiveness of synovial T cells.

### A55 T-LYMPHOCYTE CLONAL ALTERATIONS IN ANTI-CITRULLINATED PROTEIN ANTIBODY-POSITIVE SYNOVITIS

1T Cantaert, 1S Buurmond, 1R Thurfring, 2A Pallier, 2C Braud, 2P Klarenbeek, 2N de Vries, 2JP Soullou, 2PP Tak, 2D Baeten. 1Clinical Immunology and Rheumatology, Academic Medical Center/University of Amsterdam, Amsterdam, The Netherlands; 2INSERM, A22Ann Rheum Dis

**Background:** The pathophysiology of rheumatoid arthritis (RA) has been proposed to be 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-positive subset supports this hypothesis and suggests that T-cell help may be involved in the ACPA response. Therefore, we aimed to assess directly the potential involvement of T lymphocytes in ACPA-positive synovitis.

**Methods:** Synovial biopsies were obtained from actively inflamed knee joints of 54 RA patients. Of these, 37 were ACPA-positive as assessed by the anti-cyclic citrullinated peptide 2 ELISA. Synovial inflammation was assessed by standard synovial histology and immunohistochemistry. Total RNA was extracted from the biopsies of 11 ACPA-positive RA synovia, seven ACPA-negative RA synovia and 10 control spondyloarthritis (SpA) synovia with similar levels of T- and B-cell infiltration and from the peripheral blood mononuclear cells (PBMC) of seven ACPA-positive RA patients, six ACPA-RA patients and eight SpA patients. T-lymphocyte clonality in the inflamed synovium was studied by TcLandscape technology. Alterations of the normal TCR length distribution were analysed for all Vbeta families, after correction for the number of specific transcripts and using Ct as the internal control.

**Results:** Comparing ACPA-positive with ACPA-negative RA patients, there were no significant differences in demographic features, disease duration, treatment and global disease activity. At the synovial level, there was no difference in global inflammatory infiltration, synovial lining hyperplasia, vascularity and infiltration with CD3+ T cells, CD20+ B cells and CD138+ plasma cells. The TCR analysis showed no difference in the degree of alteration of the TCR length distribution (summarised for all Vbeta families) in RA synovitis compared with SpA synovitis. Subdividing RA patients into ACPA-positive and ACPA-negative patients showed a significantly higher degree of TCR alteration in ACPA-positive synovitis (29.1 ± 8.6%) compared with ACPA-negative synovitis (19.5 ± 4.6%; p = 0.011). There was also a significant difference between ACPA-positive synovial T cells and SpA synovial T-cell TCR length distribution (22.6 ± 4.2%; p = 0.012). The differences in TCR length alteration were observed in several Vbeta families and were different in each individual sample. TCR length distributions in PBMC were significantly lower than in synovial tissue for both RA (16.0 ± 3.7 vs 24.5 ± 9.0, p = 0.002) and SpA (13.9 ± 2.3 vs 22.6 ± 4.2, p<0.001), but not different from each other or healthy controls. Sequencing of selected Vbeta families confirmed that the observed alterations of the TCR length distribution corresponded to mono or oligoclonal T-cell expansions.

**Conclusion:** This study demonstrates a specific increase of T-cell clonality in ACPA-positive synovitis versus ACPA-negative RA synovitis and SpA synovitis, which is not reflected in peripheral blood. These data suggest a specific contribution of T lymphocytes in the local disease process in ACPA-positive RA.

### A56 TH17 INDUCTION BY CANDIDA ALBICANS RESULTS IN MORE DESTRUCTIVE EXPERIMENTAL ARTHRITIS

1R Marinissen, 1M Koenders, 2F van de Veerdonk, 2IM Neetea, 2J Joosten, 1W van den Berg, 1Radboud University Nijmegen Medical Centre, Rheumatology Research and Advanced Therapeutics, Nijmegen, The Netherlands; 2Radboud University Nijmegen Medical Centre, Department of Medicine, Nijmegen, The Netherlands

**Objective:** Recently, a possible role for Dectin-1 ligands derived from yeast has been described in the generation of Th17 cells via the IL-23/IL-17 axis. As the Th17 cell is regarded as a pathogenic and destructive Th cell, the objective of this study was to identify the impact of the enlarged contribution of Th17 on the chronic streptococcal cell wall (SCW) arthritis model.

**Methods:** For this purpose, different Dectin-1 ligands (yeast particles) were repeatedly co-injected with the SCW fragments into the knee joint. Therefore, we used conidia of Candida albicans and zymosan A from Saccharomyces cerevisiae. To induce chronic SCW arthritis, mice were repeatedly injected into the knee joint with cell wall fragments of Streptococcus pyogenes on days 0, 7, 14 and 21. SCW fragments were given alone or in combination with 1*10^5 conidia particles of C. albicans or 2 µg zymosan A. During the experiment, joint swelling was monitored using 99mTc measurements and the mice were killed on days 22 and 28. Quantitative PCR analysis for different proinflammatory mediators and matrix metalloproteinases (MMP) was performed on the synovium and cartilage. In addition, the levels of inflammatory cytokines were measured in synovial washouts and supernatant of anti-CD3/CD28 cultured lymphocytes of the draining lymph nodes. On day 28, joints were processed for histological analysis.

**Results:** In-vitro studies using peritoneal macrophages demonstrated that candida was very potent in inducing tumour necrosis factor, KC and macrophage inflammatory protein type 1α/β production, whereas zymosan was more potent in inducing IL-1, IL-12p70 and in particular IFNγ. Culture of human peripheral blood mononuclear cells with candida, and not zymosan, induced high Th17 numbers and strong IL-17 production. The intra-articular injection of yeast particles alone did not induce arthritis, whereas SCW arthritis was aggravated with each repeated SCW injection. The addition of yeast particles to the SCW injections slightly increased joint swelling. Histology also showed slightly enhanced joint inflammation in both the SCW/candida and the SCW/zymosan group compared with SCW arthritis alone. However, of great interest, only the combination of SCW plus candida resulted in severe chondrocyte death and enhanced destruction of cartilage and bone. IL-17 production by lymphocytes from the draining lymph nodes was significantly increased in the SCW/candida group, whereas IFNγ levels were comparable in all groups. In the arthritic joints at day 22, the addition of candida resulted in an increased expression of IL-1, tumour necrosis factor alpha, IL-6 and IL-17. In addition, the synovial messenger RNA levels from IL-23p19, IL-12p70 and in particular IFNγ. Culture of human peripheral blood mononuclear cells with candida, and not zymosan, induced high Th17 numbers and strong IL-17 production. The intra-articular injection of yeast particles alone did not induce arthritis, whereas SCW arthritis was aggravated with each repeated SCW injection. The addition of yeast particles to the SCW injections slightly increased joint swelling. Histology also showed slightly enhanced joint inflammation in both the SCW/candida and the SCW/zymosan group compared with SCW arthritis alone. However, of great interest, only the combination of SCW plus candida resulted in severe chondrocyte death and enhanced destruction of cartilage and bone. IL-17 production by lymphocytes from the draining lymph nodes was significantly increased in the SCW/candida group, whereas IFNγ levels were comparable in all groups. In the arthritic joints at day 22, the addition of candida resulted in an increased expression of IL-1, tumour necrosis factor alpha, IL-6 and IL-17. In addition, the synovial messenger RNA levels from IL-23p19, IL-22, and RORγT were upregulated in this SCW/candida group. Furthermore, we demonstrated a clear upregulation of MNF15, MMP15, MMP14, RANKL and cathepsin K in the synovium and cartilage of these mice.

**Conclusion:** These results show a clear synergy between SCW fragments and C. albicans in skewing the model further towards a Th17 profile, resulting in more destructive arthritis.
Pathogenic memory T helper cells have a stable memory for the expression of distinct effector molecules, eg, cytokines, probably making them refractory to immunosuppressive therapeutic intervention. To detect biomarkers of pathogenic memory T cells, we compared the gene expression profiles of once and repeatedly activated Th cells. The evolutionary conserved transcription factor twist1 is specifically expressed by repeatedly activated Th1 cells. Th cells isolated from the site of inflammation of immunosuppressed patients with chronic inflammatory diseases, such as rheumatic disorders or Crohn’s disease and ulcerative colitis, express high levels of twist1, whereas the twist1 levels in Th cells isolated from healthy tissue or blood are near the detection limit. In a model of antigen-induced arthritis, the transfer of repeatedly activated Th1 cells results in the induction of an inflammatory arthritis with subsequent chronic inflammation. Knock-down of twist1 in Th1 cells inducing arthritis in this model leads to drastic exacerbation of chronic inflammation compared with the transfer of control cells. In particular, infiltration of granulocytes and mononuclear cells into the inflamed tissue of the knee joint was enhanced, as were characteristics of chronic inflammation, ie, prominent hyperplasia of the lining cells, pannus formation, increased vascularisation and hyperplasia of synovial fibroblasts in the sublining layer. Anti-tumour necrosis factor alpha (TNFα) staining of joint sections revealed stronger TNFα expression, especially in the lining cells and the sublining layer after the transfer of twist1-knockdown Th1 cells. Twist1 thus acts as an endogenous regulator of Th1-induced inflammation. In Th1 cells, twist1 controls a set of various genes, mostly effector cytokines, molecules involved in migration, cell activation and in apoptosis. The expression of twist1 itself depends on STAT4 (IL-12) signalling, and is transiently induced by T-cell receptor engagement-activated nuclear factor kappa B and nuclear factor of activated T-cell transcription factors.

Twist1 and the genes controlled by it represent biomarkers and novel targets for therapies aimed at the specific elimination of pathogenic memory Th1 cells.

5. Immune regulation, cytokines, cellular interactions and molecular pathways

**ABSENCE OF A CLASSICALLY ACTIVATED MACROPHAGE CYTOKINE SIGNATURE IN PERIPHERAL Spondyloarthritis, INCLUDING PSORIATIC ARTHRITIS**

C Ambarus, T Noordenbos, B Vandereen, S Krausz, T Cantaert, N Yeremenko, M Boumans, R Lutter, P-P Tak, D Baeten. 1Department of Clinical Immunology and Rheumatology, Academic Medical Center/University of Amsterdam, Amsterdam, The Netherlands; 2Department of Internal Medicine, Ghent University, Ghent, Belgium; 3Department of Experimental Immunology, Academic Medical Center/University of Amsterdam, Amsterdam, The Netherlands

**Objectives:** Peripheral spondyloarthritides (SpA) is characterised by macrophages expressing CD163, a marker of alternative activation (AAM). Here, we assessed whether this differential infiltration with AAM versus classically activated macrophages (CAM) has functional consequences for the local inflammatory milieu.

**Patients and Methods:** The effect of SpA and rheumatoid arthritis (RA) synovial fluid (SF) on macrophage polarisation was tested on normal peripheral blood monocytes in vitro. SF levels of CAM and AAM-derived mediators were analysed by ELISA and Luminox in 47 non-psoriatic SpA, 55 RA and 15 psoriatic arthritis (PsA) patients. Paired synovial biopsies were analysed by histology.

**Results:** SpA SF preferentially promoted AAM versus CAM polarisation in vitro, even if SF levels of the prototypical AAM-polarising factors (IL-4, IL-13 and IL-10) were not increased in comparison with RA. Despite a similar degree of overall joint inflammation in SpA and RA, SpA synovitis displayed strongly reduced SF levels of CAM-derived, but not AAM-derived mediators such as tumour necrosis factor alpha (TNFα), IL-1β, IL-12 p70 and IP-10. SF levels of CAM-derived mediators correlated well with peripheral joint inflammation in RA, but neither these mediators nor IL-1α and IL-17 correlated with synovial inflammation in SpA. Of interest, the SF cytokine profile in PsA, a more destructive subtype of SpA, was perfectly similar to non-psoriatic SpA.

**Conclusion:** The local inflammatory milieu is clearly different in SpA compared with RA peripheral arthritis. Synovitis in SpA, including PsA, is characterised by a selective decrease of CAM-derived pro-inflammatory mediators such as TNFα and IL-1β.
Background and Objectives: Adipokines, cytokine-like mediators, are not only produced by adipose tissue. They also play an important role in rheumatoid arthritis (RA), which is associated with the increased production of adipokines in the serum as well as in the synovial fluid and tissue. We therefore analysed how the adipokine adiponectin affects the gene expression of several cell types involved in RA pathogenesis with the focus on RA synovial fibroblasts (RASF), aggressive cartilage invading cells. For this purpose, we were mainly interested in genes and proteins concerned with inflammation and cartilage degradation. We also performed analyses of the underlying signalling pathways.

Methods: Human RASF, lymphocytes, endothelial cells and chondrocytes were stimulated in vitro with adiponectin. Affymetrix oligonucleotide microarrays and protein arrays (RayBiotech) were used to screen for changes in gene expression of RASF. Real-time PCR was used to determine relative messenger RNA levels and immunoassays were used to quantify secreted proteins. The expression of selected genes and proteins was quantified for RASF, lymphocytes, endothelial cells and chondrocytes upon adiponectin stimulation. Signalling of adiponectin was inhibited in RASF to confirm the adiponectin-mediated effects.

Results: RASF stimulated with adiponectin were induced to synthesize distinct proinflammatory molecules (mRNA: eg, pros-taglandin-endoperoxide synthase 2, 19.9-fold), chemokines (protein: eg, MCP-1, 15.8-fold), adipokines (protein: eg, PBEF-1, 6.1-fold), growth factors (mRNA: eg, FGF-10, 5.0-fold), genes involved in bone metabolism (mRNA: eg, stanniocalcin 1, 19.8-fold) and matrix remodelling proteins (mRNA: eg, MMP-10 88.7-fold). Lymphocytes, endothelial cells and chondrocytes also responded to adiponectin stimulation, specifically with the increased secretion of cytokines and chemokines (eg, IL-6, MCP-1, RANTES). In RASF, the adiponectin-induced secretion of IL-6, MCP-1, and MMP-10 was reduced by inhibition of the key intracellular signalling molecule p38 mitogen-activated protein kinase (MAPK) as well as protein kinase C (PKC).

Conclusions: Our data show that adiponectin significantly affects the gene expression profile of RASF. The regulated genes strongly suggest that adiponectin plays an important role in promoting inflammation and matrix degradation in RA. Chemokines produced by RASF upon adiponectin stimulation potentially attract inflammatory cells to the synovium to increase inflammation further. Besides RASF, other cell types including lymphocytes, endothelial cells and chondrocytes are also affected by adiponectin in a way that promotes the progression of the disease. The hypothesis that intracellular signalling of adiponectin is mainly mediated via p38 MAPK but also PKC is supported by our data. Our data provide further insight into the molecular pathogenesis of RA and may thus help find new approaches for therapeutic intervention.
remission (seven infliximab by week 14 and three methotrexate by week 52). The other nine patients achieved only a modest reduction of disease activity (median ADAS28 <2). DAS28, C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR) and joint counts were no different at baseline between the treatment groups. They also did not predict response or remission. Naive cell frequency at baseline was markedly increased in the patients who achieved remission (p = 0.003), as well as the IRC frequency being lower (p = 0.006).

Conclusions: Remission can be achieved in early disease using both conventional and biological therapies. The immune status of RA patients at baseline can be used to predict response to both. Losing thymic-derived Treg (p = 0.125). These data suggest that patients with more profound immunological abnormalities (low naive and high IRC) were less likely to respond to treatment. We investigated the length of duration of inflammatory symptoms as a potential mechanism behind this dysregulation and found an inverse relationship with both naive cell frequency and the proportion of thymic Treg (R = -0.527 and R = -0.676, respectively).

A63 BIOLOGICAL ROLE OF MYELOID CELL-DERIVED IL-1 RECEPTOR ANTAGONIST IN COLLAGEN-INDUCED ARTHRITIS
C Lamacchia, G Palmer, D Talabot-Ayer, C Gabay, Department of Pathology and Immunology, University of Geneva School of Medicine (CMU), Geneva, Switzerland

Background: The balance between IL-1 and its specific inhibitor, IL-1 receptor antagonist (IL-1Ra), plays a major role in the development of arthritis and in the local inflammatory events leading to joint damage. In lymphoid organs, dendritic cell-derived IL-1 is required for antigen-specific T-cell activation and it has been demonstrated that IL-1Ra is involved in the control of Th17 response in arthritis. However, the contribution of myeloid cells as cellular sources of IL-1Ra in lymphoid organs has not been formally examined. In addition, activated myeloid cells, including macrophages and neutrophils, are actually considered as major sources of IL-1Ra within inflamed joints.

Objective: The aim was to define the relative role of myeloid cells compared with other sources of IL-1Ra in collagen-induced arthritis (CIA).

Methods: Conditional myeloid cell-specific IL-1Ra-deficient mice (IL-1RdeltA-M) were generated in the DBA/1 background by using the LoxP/Cre-recombinase system. CIA was induced in IL-1RdeltA-M mice and control littermates by one single immunisation with bovine type II collagen (CII) in complete Freund’s adjuvant. Arthritis severity was assessed by clinical scoring. Histological analysis of the joints at the end of the experiment (day 25) was used to confirm the clinical assessments of arthritis. The levels of different cytokines were quantified by ELISA.

Results: IL-1RdeltA mice exhibited early disease onset, starting at day 11 after immunisation and a severe form of CIA. In contrast, lower disease incidence with later onset (day 18) and lower severity of articular inflammation were observed in control mice after one single immunisation with CII. Consistent with clinical findings, inflammation, cartilage erosion and neutrophil infiltration in IL-1RdeltA mice were markedly increased compared with control mice. The ex-vivo proliferation of draining lymph node (DLN) cells to CII was significantly increased in cells isolated from IL-1RdeltA mice compared with control mice, suggesting a higher sensitivity to CII in conditional knockout mice. Interestingly, IFN-γ production was significantly enhanced and IL-17 production tended to be increased in CII-stimulated DLN cells isolated from IL-1RdeltA mice compared with control mice. Surprisingly, in spite of myeloid cell-derived IL-1Ra deficiency, IL-1Ra levels in arthritic joints of IL-1RdeltA mice were significantly higher than in joints of control mice.

Conclusions: The results suggest that myeloid cell-derived IL-1Ra plays a major role in the control of the immune response and in both the development and severity of CIA. We propose that excess IL-1 signalling in DLN, due to IL-1Ra deficiency, leads to enhanced IFN-γ and IL-17 production, which may in turn contribute to the enhanced severity of arthritis. The results suggest that resident cells such as chondrocytes and synovial fibroblasts contribute to the local production of IL-1Ra in arthritic joints of IL-1RdeltA mice.

A64 BLOCKADE OF THE HIGH AFFINITY IL-7 RECEPTOR INHIBITS PROTEOGLYCAN-INDUCED ARTHRITIS

1 J van Roon, S Hartgring, F Broeze, W van Eden, J Bijlsma, C Willis, F Lafeber. Department of Rheumatology and Clinical Immunology, UMC Utrecht, Utrecht, The Netherlands; Amgen, Seattle, Washington, USA; Department of Immunology, Vet Fac, Utrecht University, Utrecht, The Netherlands

Purpose: Apart from its role in T-cell development, IL-7 is known to stimulate mature T cells. It also induces T-cell-dependent activation of monocytes, B cells and osteoclasts. High levels of this immunostimulatory cytokine are found in several arthritic conditions including rheumatoid arthritis (RA). IL-7 effects are mediated through the high affinity IL-7 receptor alpha chain (IL-7R) in conjunction with the common gamma chain. We propose that excess IL-1 signalling in DLN, due to IL-1Ra deficiency, leads to enhanced IFN-γ and IL-17 production, which may in turn contribute to the enhanced severity of arthritis. The results suggest that resident cells such as chondrocytes and synovial fibroblasts contribute to the local production of IL-1Ra in arthritic joints of IL-1RdeltA mice.

Methods: Proteoglycan-induced arthritis was initiated by immunising aged female Balb/c mice with human proteoglycan. Approximately one week after the second immunisation mice developed arthritis. Mice (n = 25) were treated with phosphate-buffered saline (PBS), as a control, or with an anti-IL-7R antibody (either 100 µg or 500 µg on experimental days 21, 23, 26, 30 and 33). Arthritis severity and incidence were determined daily by visual examination of swelling and redness of the paws (maximum score of four/paw; 16/mouse). Furthermore, cell numbers from thymus and spleen, and numbers of CD4, CD8 and CD25 T cells were assessed and the expression levels of IL-7R on T cells were determined.

Results: Arthritis developed in 92% of the animals in the PBS-treated group (mean score 6.5 ± 0.9). Anti-IL-7R treatment (100 µg) significantly reduced arthritis, compared with PBS-treated animals, from experimental days 25 to 35 (mean arthritis inhibition 58%, p<0.05 at all time points). The higher dose anti-IL-7R (500 µg) also showed significant arthritis suppression, although less outspoken than the lower dose. Arthritis incidence of the mice treated...
with 100 μg anti-IL-7R was significantly diminished (on day 35, 54% vs 92% in the PBS group). Isotype control antibody did not have any significant effects on arthritis. Thymic development, measured from the absolute numbers of thymocytes and thymocyte subsets, was not different in anti-IL-7R-treated mice compared with PBS-treated mice. Peripheral splenic CD4 and CD8 T cells were modestly decreased by treatment with 500 μg anti-IL-7R (~20% and ~52%, respectively, p<0.05) compared with PBS. Treatment with 100 μg anti-IL-7R resulted in a limited decrease in the numbers of splenic CD8 T cells (~18%, p<0.05), not of CD4 T cells. No significant changes in CD25 regulatory T cells were observed. In addition, significant reductions in IL-7R expression levels on both CD4 and CD8 T cells were found (in the 100 μg group; mean fluorescence intensity ~24% and ~20%; respectively).

Conclusions: Our data show that blockade of IL-7R potently inhibits the development of arthritis associated with only limited T-cell reduction in aged mice. This study reveals an important role for IL-7R-expressing cells in arthritis initiation and indicates that IL-7R targeting should be further evaluated as a new treatment strategy for RA.

**A65 EARLY TREATMENT AND IMMUNOLOGICAL REMISSION PREDICT LONG-TERM BIOLOGICAL-FREE REMISSION POST-TUMOUR NECROSIS FACTOR BLOCKERS**

B Saleem, R Parmar, V Goeb, P Conaghan, P Emery, F Ponchel. LVMM, Section MSK, University of Leeds, Leeds, UK

Objectives: Stopping biologicals while maintaining response is now the main objective of therapy in rheumatoid arthritis (RA). A sustained clinical remission (disease activity score (DAS28) <2.6) has been observed after the cessation of tumour necrosis factor (TNF) blocker therapy in RA patients; however, only with early disease. Naïve T cells are lost early in disease, and an abnormal T-cell subset inflammation-related cells (IRC) with potential pathological activity has been described. In disease-modifying antirheumatic drug-induced remission, IRC levels predicted relapse. Other studies have shown that reduced regulatory T cell (Treg) function in RA can normalise in patients treated with infliximab. This study aimed to determine whether the immunological status of patients in remission could be used to predict outcome after stopping therapy.

Methods: Advanced six-colour flow cytometry was used to measure the frequency of IRC, naïve CD4 T cells and thymus-derived Treg (CD4+CD25highFoxp3+CD62L+) in 27 early (<24 months) RA patients achieving clinical remission (DAS28 <2.6 for at least 6 months) following 12 months of TNF blockade (adalimumab n = 16 and infliximab n = 11). The primary outcome was sustained remission or flare (defined as an increase of 1.2 in DAS28 or as a DAS28 >2.6).

Results: At 18 months 17 patients had sustained remission and 10 had flared. No significant difference was found between groups for demographic or clinical data (DAS28, C-reactive protein, anti-cyclic citrullinated peptide or rheumatoid factor titres, joint counts); however, symptom duration before TNF-blocker therapy (p = 0.007, median 5.5 vs 12 months) and total duration of active disease (symptom plus time on treatment until remission, p = 0.043, median 10 vs 18 months) were shorter in patients with sustained remission. Immunological parameters also showed significance with higher naïve cell frequency (p = 0.007) and lower IRC (p = 0.001) in sustained remission, suggesting the recovery of thymic activity and control over inflammation-driven T-cell differentiation. Surprisingly, a higher frequency of CD25highFoxp3+ Treg (p<0.001) was associated with flare. Analysis of Treg with regard to their thymic origin (expression of CD62L) showed a highly significant difference in the phenotype of Treg between sustained remission and flare (p = 0.005, median 70 vs 43% CD62L+Treg). However, no difference was found in CD62L+Treg frequencies. As already described, CD25highFoxp3+ may also represent recently activated T cells. Binary logistic regression showed that short duration of symptoms before treatment is the main predictor of long-term remission with 94% accuracy.

Conclusion: Sustained remission following cessation of TNF blockade is an achievable goal in early RA. This requires, however, early diagnosis and treatment enabling the achievement of immunological remission.

**A66 OESTROGEN RECEPTOR-ALPHA AND OESTROGEN RECEPTOR-BETA ARE EXPRESSED IN NORMAL SALIVARY EPITHELIUM AND MEDIATE THE DOWNREGULATION OF INTERCELLULAR ADHESION MOLECULE TYPE 1 INDUCED BY INTERFERON GAMMA**

1M Tinti, 1E Kassi, 1P Korkolopoulou, 1E Kapsogeorgou, 1P Moutsatsou, 1E Patsouris, 1MN Manoussakis. 1Department of Pathophysiology, National and Kapodistrian University of Athens, Athens, Greece; 2Department of Biological Chemistry, National and Kapodistrian University of Athens, Athens, Greece; 3Department of Pathology, National and Kapodistrian University of Athens, Athens, Greece

Background and Objectives: Several lines of evidence indicate the immunological role of salivary gland epithelial cells (SGEC), as well as the involvement of these cells in the pathogenesis of Sjögren’s syndrome (SS). Oestrogen deficiency also appears to be involved in SS pathogenesis, as suggested by the strong predilection of the disorder for women around menopause and by oestrogen-depleted animal models. However, the expression and functionality of the oestrogen receptors ERα and ERβ in normal human salivary epithelium is unknown. In this study, to address whether non-tumorous SGEC represent cellular targets of oestrogens, we assessed the expression and functionality of ERα and ERβ proteins in non-neoplastic minor salivary gland (MSG) tissues and cultured non-neoplastic SGEC lines.

Materials and Methods: Normal MSG biopsy specimens were obtained from individuals who underwent diagnostic biopsy for subjective sicca complaints, but presented no histopathological or clinico-oculoradiological evidence of exocrinopathy. Cultured non-neoplastic SGEC lines were established from the above normal tissues. MSG specimens and cultured non-neoplastic SGEC lines were analysed by immunohistochemistry and immunoblotting for the expression of ERα and ERβ (ERβ1 and ERβ2-isofom) proteins. The response of non-neoplastic SGEC lines to treatment with 17β-oestradiol (E2, 10⁻⁷ mol), propylpyrazole triol (PPT; ERα-selective-agonist, 10⁻¹⁰ to 10⁻⁷ mol) or diarylpropionitrile (DPN, ERβ-selective-agonist, 10⁻¹⁰ to 10⁻⁷ mol) was evaluated by flow cytometry analyses of the modulation of the expression of constitutive and IFNγ-induced intercellular adhesion molecule type 1 (ICAM-1). The effects of E2, PPT and DPN on the proliferation and viability of SGEC were assessed by the MTT and annexinV-binding assays, respectively.

Results: ERα, ERβ1 and ERβ2-isofrom proteins were detected by immunohistochemistry in the SGEC of MSG tissues and in cultured SGEC. ERα, ERβ1 and ERβ2 exhibited similar staining patterns and intensity and immunolocalised in both the nucleus and the cytoplasm of SGEC. The expression of oestrogen receptor proteins was also verified by the immunoblotting of the full-length-ERα, the ERα-A5 isoform, of the long and short forms of ERβ1 and the long form of ERβ2 in cellular extracts of cultured SGEC. Cultured SGEC were found to respond to E2, PPT and DPN as indicated by the strong inhibition of IFNγ-induced ICAM-1 expression (E2: p = 0.01, PPT and DPN: both for p = 0.05). Treatment with E2, PPT or DPN did not have any effect on constitutive ICAM-1 expression and was not found to induce growth or apoptosis in SGEC.

Conclusion: Our findings indicate that SGEC express functional ERα and ERβ. The detection of multiple isoforms of ERα and ERβ...
on SGEC probably indicates the complexity of oestrogenic signalling in these cells. 17β-estradiol and both ERα and ERβ-selective agonists were found to mediate the inhibition of IFNY-induced ICAM-1 expression, a fact that possibly illustrates the immunomodulatory influence of oestrogens on salivary epithelium.

**A67 EFFECT OF ABATACEPT ON REGULATORY T CELLS AND ANTIGEN-PRESENTING CELLS IN PATIENTS WITH RHEUMATOID ARTHRITIS**

**Introduction:** Abatacept represents a soluble recombinant human fusion protein comprising the extracellular domain of human CTLA4 and a fragment of the Fc domain of human IgG1 (CTLA4-Ig). As a result of the interaction with CD80/CD86, abatacept inhibits the binding of CD28 and thereby effector T-cell activation. In addition, however, this interaction might also have potentially unwanted effects on regulatory T cells (Treg) and/or antigen-presenting cells (APC). We therefore performed phenotypic and functional analysis of peripheral blood mononuclear cells (PBMC) in rheumatoid arthritis (RA) patients before and after the initiation of abatacept therapy.

**Methods:** PBMC were collected from RA patients who fulfilled at least four criteria of the American College of Rheumatology (ACR) before and at different time points during abatacept therapy. PBMC were analysed for proportions and phenotypic characteristics of CD4+CD25+Foxp3+ Treg, CD4 and CD8 T cells, B cells, CD14+ monocytes and dendritic cells. In addition, isolated CD4+CD25high T cells were analysed for their capacity to suppress T-cell proliferation and IFNγ production.

**Results:** Proportions of CD4+CD25+Foxp3+ T cells increased upon abatacept treatment on average twofold. In addition, we observed a substantial increase in proportions of CD14+ monocytes and CD19+B cells in the majority of treated patients. Unexpectedly, however, abatacept treatment did not diminish the proliferative response of CD4 T cells upon stimulation with anti-CD8 antibody, but rather enhanced T-cell proliferation up to threefold. On the other hand, a decrease in the suppressive capacity of isolated Treg was observed. Until now no correlation of a diminished suppressive capacity with the clinical treatment response was apparent. Preliminary data also suggest effects on the expression of co-stimulatory molecules CD80 and CD86 on APC.

**Conclusion:** Until now our data suggest that abatacept not only influences effector T-cell populations in RA but in addition exerts unwanted effects on regulatory T cells (Treg) and/or antigen-presenting cells (APC). We therefore performed phenotypic and functional analysis of peripheral blood mononuclear cells (PBMC) in rheumatoid arthritis (RA) patients before and after the initiation of abatacept therapy.

**A68 EFFECTS OF TUMOUR NECROSIS FACTOR BLOCKADE BY INFlixIMAB ON THE INFLAMMASOME COMPONENT NALP3 IN RHEUMATOID ARTHRITIS PATIENTS**

**Background:** The NALP3 inflammasome is a highly conserved response system, composed of a proinflammatory protein complex involved in IL-1β production, through proteolytic cleavage by caspase-1 (IL-1β-converting enzyme). The proinflammatory cytokines IL-1β and tumour necrosis factor (TNF) are key molecules in the pathogenesis of rheumatoid arthritis (RA). TNF blockade has profound therapeutic effects in RA, with marked benefit to a majority of patients. We investigated the expression of NALP3 messenger RNA in RA patients and effects of TNF blockade by infliximab on this pathway. Preliminary findings indicate that components of the NALP3 inflammasome are modulated in infliximab-treated patients. It has also been shown that NALP3 promoter sequence variants may influence the disease state in a number of inflammatory diseases. Therefore, in addition to our NALP3 expression study, we characterised the promoter region and sequence variants of this gene (NALP3) in RA patients receiving infliximab.

**Methods:** RA patients studied had failed at least two disease-modifying antirheumatic drugs before being initiated onto infliximab. Inflammasome activation was measured by caspase-1 ELISA kit. mRNA analysis was on peripheral blood mononuclear cells at baseline from infliximab-treated patients, later classified as being good (n = 20) or non-responders (n = 15). The effect of infliximab on mRNA levels of inflammasome components, NALP3, ASC and pyrin, was measured at weeks 0, 2 and 14 of therapy (n = 20), by quantitative PCR methods. DNA regions upstream of the two NLRP3 transcription start sites were analysed for putative sequence variants, in both responders (n = 20) and non-responders (n = 10).

**Results:** RA patients have significantly higher caspase-1 activation than healthy controls. Patients who responded to infliximab had significantly lower NALP3 transcripts (p = 0.02) before the start of treatment. NALP3 mRNA levels then declined further, with significant decreases observed at both pre-RNA (p = 0.007) and post-infusion (p = 0.03), at week 14 of therapy. Inflammasin had no marked effects on NALP3 expression in non-responding patients. Sequencing of the NALP3 promoter identified five variants, already reported in cryopyrinopathy patients, and we also confirmed four of the eight sequence variations reported in the National Center for Biotechnology Information single nucleotide polymorphism database. Preliminary data suggest weak associations between three promoter polymorphisms (alleles 1750, 1789 and rs12079994) and response to treatment.

**Conclusions:** Previously reported effects of infliximab on NALP3 mRNA levels in RA patients have been confirmed, with lower NALP3 mRNA levels before initiation of therapy in patients later classified as “responders”, and these levels were further reduced during infliximab treatment. These data suggest that the NALP3 inflammasome is modulated by infliximab, with a decrease in NALP3 transcript levels in patients who respond to treatment. NALP3 is not a marker of inflammation as there was no correlation with C-reactive protein levels; therefore, a lower pre-therapy level of NALP3 may be a biomarker of a beneficial response to infliximab.

**A69 IL-17 SYNERGY WITH TUMOUR NECROSIS FACTOR CAUSES STRIKING CARTILAGE EROSION IN VIVO**

**Background:** IL-17 is a proinflammatory T-cell cytokine that contributes to inflammation and destruction in rheumatoid arthritis (RA). IL-17 may aggravate experimental arthritis independent of tumour necrosis factor (TNF) and IL-1. On the other hand, in-vitro studies have shown that IL-17 can synergise with TNF, resulting in enhanced cytokine and chemokine production.

To study the combined effects of TNF and IL-17 on joint inflammation and destruction in vivo, mice were intra-articularly injected into the knee joint with adenoviruses encoding for murine TNFα and IL-17.

Local overexpression of either TNF or IL-17 alone causes synovial inflammation and reversible cartilage proteoglycan depletion. This modest joint pathology was clearly exaggerated by combining TNF and IL-17. Interestingly, only in the combination group were severe chondrocyte death and cartilage surface erosions found, indicating synergy between TNF and IL-17 on irreversible cartilage destruction.
To exclude the possibility that this enhanced cartilage destruction was merely the result of increased inflammation, we lowered the dose in the combination group to obtain similar inflammation as with the single viruses. Despite similar cell influx in the three different groups, severe cartilage destruction was still found with the combination of TNF and IL-17. Overexpression of TNF plus IL-17 significantly aggravated cartilage VDIPEN expression, a marker for matrix metalloproteinase (MMP)-driven cleavage of aggrecan.

To study the effect of TNF and IL-17 on the expression of MMP and tissue inhibitors of metalloproteinases, quantitative PCR analysis was performed. In both synovium and cartilage, MMP13 and MMP14 were synergistically upregulated by the combination of TNF and IL-17. In addition, MMP9 in synovium and MMP3 in cartilage were synergistically increased by these two cytokines. In the combination group with the lower dose (and similar cell influx as TNF or IL-17 alone), the genes that were still upregulated in this group compared with TNF or IL-17 alone were MMP5 and MMP13 in the cartilage. This chondrocyte-specific MMP upregulation may well explain the increased VDIPEN expression found on immuno-histochemistry.

In conclusion, the synergy between TNF and IL-17 in vivo results in a limited increase of bone erosion, but striking exaggeration of cartilage erosion and chondrocyte death, in line with the synergistic upregulation of erosive enzymes such as MMP3 and MMP13.

Conclusions: The higher concentration of IL-4 in the EA group probably results from the induction of its synthesis by proinflammatory cytokines. The correlation between IL-4 and the number of platelets may be explained by megakaryocyte progenitor line stimulation by this cytokine. Higher activity of STAT in the peripheral blood leucocytes of EA patients may confirm the hypothesis of the importance of STAT-mediated signalling in EA pathogenesis. No correlation between STAT-6 and IL-4 explained the impaired cell response to IL-4 in RA. The negative correlation between STAT-3 and IL-4 is probably connected with antagonistic function of IL-4 and IL-6, which acts via STAT-3 and is present in high concentrations in EA (as already shown).

A70 IL-4 CONCENTRATION AND STAT ACTIVATION IN EARLY ARTHRITIS

Background and Objectives: Early arthritis (EA) is an inflammation of the joints, which may evolve into rheumatoid arthritis (RA) or other rheumatic diseases. During EA inflammation is in the acute phase and could possibly be stopped if the exact pathogenetic mechanism was unravelled. It is known that in RA cell response to IL-4 is impaired. IL-4 acts via the JAK/STAT pathway (mainly STAT-6). The study was undertaken to investigate the relation between serum IL-4 concentrations and STAT activation in EA.

Materials and Methods: 51 patients (55% male) diagnosed with EA and 30 (33% male) healthy individuals as controls have been recruited. The duration of symptoms was limited to 12 months and patients were not treated with disease-modifying antirheumatic drugs apart from sulfasalazine. A 6-month-long follow-up was performed to check for the final diagnosis. Immunocytochemistry was used to examine the presence of active STAT-3 and STAT-6 in EA.

Results: In the EA group the mean age was 44.1 years and the mean disease activity score (DAS28) was 5.2. 12 patients (39%) were diagnosed with RA, 11 patients (35%) with undifferentiated arthritis (UA), three patients (10%) with reactive arthritis, two patients (6%) with paraneoplastic syndrome, two patients (6%) with gout and one patient (3%) with ankylosing spondylitis. In the control group the mean age was 32.4 years. The mean concentration of IL-4 on the first visit was higher (p<0.05) in the EA group (10.0 pg/ml) compared with controls (3.14 pg/ml) and after follow-up significantly decreased (5.20 pg/ml). The mean percentage of STAT-3 and STAT-6-positive cells on the first visit was higher (p<0.05) for the EA patients (31.4 and 34.1, respectively) compared with controls (21.6 and 22.6, respectively). There was no difference in STAT activation between EA (25.6 and 28.3, respectively) and the control group on the second visit. In the UA subgroup on the first visit IL-4 negatively correlated with STAT-3 activation (RP = -0.64). Moreover, in the EA group on the second visit the IL-4 concentration correlated with the number of platelets (RF 0.52).

Conclusions: IL-7 concentration and STAT activation in early arthritis could explain the increased VDIPEN expression found on immunohistochemistry. In conclusion, the synergy between TNF and IL-17 in vivo results in a limited increase of bone erosion, but striking exaggeration of cartilage erosion and chondrocyte death, in line with the synergistic upregulation of erosive enzymes such as MMP3 and MMP13.
Cytokine memory of T helper lymphocytes is crucial for protection against recurring pathogens but is also the driving force of chronic inflammation. Deciphering the molecular mechanism leading to the stability of cytokine memory may lead to the design of therapeutic strategies in chronic inflammatory immune disorders. We have focussed our studies on the memory for IL-17 expression in Th17 cells, shown to play an essential role in the defence against extracellular bacteria and fungi, but also in the pathogenesis of autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis. We have developed a cytometric IL-17 secretion assay for the isolation of viable Th cells secreting IL-17, which allows us to study the memory for IL-17 expression in Th17 cells on the single cell level. When differentiated in vitro from naive Th lymphocytes according to state-of-the-art protocols by activation in the presence of transforming growth factor beta (TGFβ), IL-6 and IL-23, isolated IL-17-expressing Th17 cells did not re-express IL-17 during later reactivation in the absence of the original instructive signals. Instead, in-vitro-generated Th17 cells could be converted to Th1 or Th2 cells with IL-12 or IL-4, respectively. In contrast, IL-17-expressing Th cells, which were generated in vivo and isolated directly ex vivo maintained IL-17 expression upon in-vitro culture and were refractory to Th1 or Th2 polarising signals. Th17 memory cells when adoptively transferred potently induced joint inflammation in a model of ovalbumin-induced arthritis, demonstrating that Th17 cells are indeed pathogenic in a model of rheumatoid arthritis.

**A73 INFLAMMASOME COMPONENT NALP3 IN RHEUMATOID ARTHRITIS PATIENTS PREDICTS RESPONSE AND IS MODULATED BY SUCCESSFUL INFlixIMAB THERAPY**

1RJ Mathews, 1SJ Churchman, 1JR Couthard, 1LD Church, 1S Savic, 1C Wong, 1D Bryer, 1M Buch, 1GP Cook, 1P Emery, 1MF McDermott. *Section of Musculoskeletal Disease, Leeds Institute of Molecular Medicine, Leeds, UK; 1Leeds Institute of Molecular Medicine, Leeds, UK*

**Background:** The NALP3 inflammasome is a highly conserved response system, composed of a proinflammatory protein complex involved in IL-1β production, through proteolytic cleavage by caspase-1 (IL-1β-converting enzyme). The proinflammatory cytokines IL-1β and tumour necrosis factor (TNF) are key molecules in the pathogenesis of rheumatoid arthritis (RA). TNF blockade has profound therapeutic effects in RA, with marked benefit to a majority of patients. We investigated the expression of NALP3 messenger RNA in RA patients and the effects of TNF blockade by infliximab on this pathway. Preliminary findings indicate that components of the NALP3 inflammasome are modulated in infliximab-treated patients. It has also been shown that NALP3 promoter sequence variants may influence the disease state in a number of inflammatory diseases. Therefore, in addition to our NALP3 expression study, we characterised the promoter region and sequence variants of this gene (NLRP3) in RA patients receiving infliximab.

**Methods:** RA patients studied had failed at least two disease-modifying antirheumatic drugs, before being initiated onto infliximab. Inflammasome activation was measured by a caspase-1 ELISA kit. mRNA analysis was on peripheral blood mononuclear cells at baseline from infliximab-treated patients, later classified as being good (n = 20) or non-responders (n = 15). The effect of infliximab on mRNA levels of inflammasome components, NALP3, ASC and pyrin, was measured at weeks 0, 2 and 14 of therapy (n = 20), by quantitative PCR methods. DNA regions upstream of the two NLRP3 transcription start sites were analysed for putative sequence variants, in both responders (n = 20) and non-responders (n = 10).

**Results:** RA patients have significantly higher caspase-1 activation than healthy controls. Patients who responded to infliximab had significantly lower NALP3 transcripts (p = 0.02) before the start of treatment. NALP3 mRNA levels then declined further, with significant decreases observed at both pre (p = 0.007) and post-infusion (p = 0.05) at week 14 of therapy. Infliximab had no marked effects on NALP3 expression in non-responding patients. Sequencing of the NALP3 promoter identified five variants, already reported in cryopyrinopathy patients, and we also confirmed four of the eight sequence variations reported in the National Center for Biotechnology Information single nucleotide polymorphism database. Preliminary data suggest weak associations between three promoter polymorphisms (alleles 1750, 1789 and rs12079994) and response to treatment.

**Conclusions:** Previously reported effects of infliximab on NALP3 mRNA levels in RA patients have been confirmed, with lower NALP3 mRNA levels before initiation of therapy in patients later classified as “responders”, with these levels being further reduced during infliximab therapy. These data suggest that the NALP3 inflammasome is modulated by infliximab, with a decrease in NALP3 transcript levels in patients who respond to treatment. NALP3 is not a marker of inflammation, as there was no correlation with C-reactive protein levels; therefore, a lower pretherapy level of NALP3 may be a biomarker of a beneficial response to infliximab.

**A74 MESENCHYMAL STEM CELLS INHIBIT HUMAN TH17 CELL DIFFERENTIATION VIA THE INDUCTION OF IL-10-PRODUCING T-REGULATORY CELLS**

S Ghanam, J Pene, C Jorgensen, H Yssel. *Insamr UB44, Hôpital St Eloi, Montpellier, France*

Mesenchymal stem cells (MSC) are not only precursors of various tissues, but also possess immunomodulatory properties and therefore may play a role in the maintenance of peripheral tolerance and autoimmunity. Recently, a novel T-cell population, called Th17 cells, has been identified that plays an important role in the pathogenesis of inflammatory and autoimmune diseases, such as rheumatoid arthritis, via the production of a variety of pro-inflammatory cytokines and chemokines. It has been reported that MSC are able to suppress alloantigen or anti-CD3/anti-CD28 monoclonal antibody-induced T-lymphocyte activation and proliferation in vitro.

Therefore, in the present study, we investigated the immunomodulatory effects of human bone marrow-derived MSC on the in-vitro differentiation of Th17 cells by analysing the production of IL-17 and other pro-inflammatory cytokines by these cells. Moreover, the effect of MCS on the cytokine production profile of fully differentiated Th17 cells was analysed.

Culture of CD4, naive, cord blood T cells in the presence of IL-1β, IL-6, IL-23 and transforming growth factor beta 1 resulted in the emergence of a significant percentage of T cells expressing CCR6, a chemokine receptor specifically expressed by Th17 cells, as well as in the production of the pro-inflammatory cytokines IL-22, IL-17, oncostatin M and IFNγ.

Under these experimental conditions, the presence of MSC prevented the emergence of CCR6-expressing Th17 cells and strongly inhibited the production of all pro-inflammatory cytokines produced by these cells. In contrast, MCS strongly induced the production of the regulatory cytokine IL-10 and, importantly, induced the expression of the T regulatory cell-specific transcription factor Foxp3 in both differentiating and fully differentiated Th17 cells.

Taken together, these results show that human MSC have anti-inflammatory effects on CD4 T cells by inhibiting T-cell differentiation along the Th17 pathway, via the modulation of the cytokine production profile of both differentiating and fully
differentiated T cells, thereby resulting in the induction of a T-regulatory cell phenotype.

### A75 NORMALISATION OF THE IMMUNOLOGICAL STATUS OF EARLY RHEUMATOID ARTHRITIS PATIENTS PREDICTS LONG-TERM REMISSION AFTER TUMOUR NECROSIS FACTOR BLOCKADE

F Ponchel, R Parmar, V Goeb, B Saleem, M Quinn, P Conaghan, P Emery. LIMM, Section MSK, LMBRU, University of Leeds, Leeds, UK

**Objective:** In early rheumatoid arthritis (RA) patients (<12 months symptoms), biological-free clinical remission (disease activity score (DAS28) <2.6) lasting up to 3 years can be induced by infliximab combined with methotrexate. In established disease, remission can also be achieved, however, not drug free. The frequency and functional capacities of T-regulatory cells (Treg) are reduced in early RA and we described a specific subset of T cells, in which differentiation is driven by inflammation (inflammation-related cells; IRC). We hypothesised that the immunological status of early untreated RA patients can be normalised and that clinical remission can be associated with this immunological remission defined using the frequency of naive and IRC CD4 T cells and Treg.

**Methods:** We used blood samples from patients included in a trial of early RA comparing infliximab plus methotrexate (n = 9) with methotrexate alone (n = 10). The clinical outcome has been reported previously (Quinn et al., 2005; A&R). Treg, IRC and naive cell frequencies were analysed longitudinally using advanced five-colour flow cytometry. Treg were defined as CD4+CD25highFoxp3+ cells and CD62L+ when emerging from the thymus. Naive and memory CD4 T cells were defined using CD3, CD4 CD45RB, CD45RA and CD62L

**Results:** The infliximab plus methotrexate group achieved remission by 14 weeks for seven of nine patients, sustained it for 12 months (median DAS28 2.08) and for up to 3 years after the trial for five of them. The control group (methotrexate) achieved only a modest reduction of disease activity by 14 weeks (average DAS28 4.25) and only three patients approached DAS28 remission at week 52, one achieving it. DAS28, CRP, Treg, IRC or naive cell frequencies were no different at baseline between groups. As soon as week 14 in the infliximab plus methotrexate group, there was a trend towards lower IRC and a higher frequency of Treg, which became significant by week 38 (p<0.03) also seen for naive cells (p = 0.057). Thymic Treg were also higher in proportion in the infliximab group (p = 0.042 at week 38). IRC were similarly reduced (p = 0.057). Thymic Treg were also higher in proportion in the infliximab group (p = 0.042 at week 38). IRC were similarly reduced (p = 0.057). Thymic Treg were also higher in proportion in the infliximab group (p = 0.042 at week 38). IRC were similarly reduced (p = 0.057). Thymic Treg were also higher in proportion in the infliximab group (p = 0.042 at week 38).

**Conclusions:** Immunological remission defined by a lowering frequency of IRC and increasing frequencies of naive and Treg CD4 T cells can be associated with clinical remission. The quality of this immunological remission appears to be better post-infliximab and to depend on the recovery of thymic function for both naive cells and Treg. As infliximab was the only tumour necrosis factor (TNF) blocker agent reported to affect Treg this study suggests that infliximab also has an important effect on thymic function and will require extension and assessment of different anti-TNF drugs.

### A76 POLYMORPHONUCLEAR NEUTROPHILS PARTICIPATE IN THE INITIATION OF COLLAGEN-INDUCED ARTHRITIS

E Rath, R Byrne, M Bonelli, A Savitskaya, B Niederreiter, JS Smolen, C Scheinecker. Division of Rheumatology, Department of Internal Medicine III, Medical University of Vienna, Vienna, Austria

**Background and Objectives:** Polymorphonuclear neutrophils (PMN) seem to participate in the initiation of rheumatoid arthritis (RA), but their precise role in the development of the synovial inflammatory response is only partly understood. We therefore analysed the initial steps of synovial inflammation in the mouse model of collagen-induced arthritis (CIA) with regard to the role of PMN.

**Materials and Methods:** CIA was induced in C57/BL6 mice by immunisation with chicken type II (CII) collagen in Freund’s complete adjuvant (CFA) at the base of the tail. On day 21, a second injection of CII in CFA was administered. Mice were monitored for clinical signs of arthritis (three times a week and tested for anti-CII serum antibodies by ELISA. Mice were killed on days 10 and 20 after the first immunisation before clinical signs of arthritis occurred and on days 1, 4, 7, 9, 11, 15, 18 and 25 after the onset of arthritis. Hind and front paws were collected and non-decalcifying cryostat sections as well as conventional paraffin sections were prepared. Joint sections were analysed after immunohistochemistry (haematoxylin and eosin, tartrate-resistant acid phosphatase, toluidine blue, Neut/4-granulocyte marker) and immunofluorescence double staining (Neu7/4, complement component C3a, MHC class II, CD11c) by confocal laser scanning microscopy.

**Results:** Mice developed clinical signs of arthritis 38 ± 7 days after the first immunisation with type II collagen. Severe periarticular and intra-articular neutrophilic infiltrates together with cartilage destruction were already detectable on the first day of clinical arthritis. In addition, strong staining of complement factor C3a was detectable in areas of inflammation and in the bone marrow of diseased animals. The extent of infiltrates did not substantially increase with time after the onset of arthritis but correlated with the degree of joint swelling. No histological signs of inflammation were detectable before the development of the clinical signs of arthritis. In addition, no C3a deposits were detectable before disease onset, suggesting that complement activation concurs with the influx of PMN. Double stainings revealed an upregulation of MHC class II on Neu7/4-positive neutrophils in areas of inflammation.

**Conclusions:** Periarticular neutrophilic infiltrates are very prominent in CIA and are already detectable in the very early stages of clinical disease. No histological signs of inflammation, especially complement activation, preceded clinical signs of arthritis. Neutrophils in inflamed areas displayed MHC class II expression, which might contribute to their functional capacities. Ongoing experiments have been designed to determine mechanism(s) that control the migratory behaviour and function of PMN in CIA as well as strategies to target PMN in therapeutic settings.

### A77 RHEUMATOID FACTOR INTERFERENCE WITH BEAD-BASED MULTIPLEX IMMUNOASSAYS PRECLUDES ITS USE IN RHEUMATOID ARTHRITIS

S Churchman, E Horner, L Kozara, LD Church, R Parmar, F Ponchel. Leeds Institute of Molecular Medicine, Section of Musculoskeletal Disease, University of Leeds, Leeds, UK

**Background:** The detection of cytokines using bead-based immunoassays became a cutting-edge technology; however, interference by rheumatoid factor (RF) with those assays has been reported. Data review on IL-7 levels in rheumatoid arthritis (RA) reported abnormally high values obtained using single and multiplex assays, respectively, compared with ELISA. Our aim was to establish whether RF is responsible for these observations and whether RF removal would allow the use of this technology.

**Methods:** Sera from healthy controls and RA patients (wide range of RF titres: 0–4500 IU RF/ml), with known IL-7 levels (measured by high sensitivity ELISA; R&D Systems, Minneapolis, MN, USA) ranging from 3.5 to 16 pg/ml, were selected. Single IL-7-plex and 25-plex (combining cytokines, chemokines and growth factors) bead assays (Biorad, Hercules, CA, USA) were used. Three methods of RF removal were tested; Sepharose-L beads (Pierce, Rockford, IL, USA), PEG 6000 and serum blocking solution. RF was measured using a
Serodia RA kit. A subset of sera were further analysed using a proteome profiler array, panel A (R&D Systems).

Results: Data obtained from healthy individuals showed a high correlation and reproducibility between ELISA and multiplex bead immunoassays (n = 10, singleplex, R = 0.844, p < 0.001). IL-7 in 10 RA patients, when measured by singleplex bead assay, showed a direct relationship with the RF titre (R = 0.666, p < 0.001); however, no such relation was seen using ELISA. Data obtained by both methods were also unrelated, with the exception of RF-negative samples. RF removal was efficient in samples with low RF titres (RF <50 IU RF/ml) but relative in others with a maximum 50% reduction in samples with an initial RF titre greater than 100 IU RF/ml. ELISA results showed minimal variation with regard to the RF removal method. In contrast, there was a minimal improvement in the bead assay measures of IL-7, which remain related to residual RF. Similar results were obtained using 17 RA serum samples and a 25-plex using the PEG 6000 removal method. Interferon, tumour necrosis factor, IL-2, IL-10 and others (with the single exception of RANTES), still showed direct relationships with RF.

Conclusion: According to our results, multiplex bead immunoassay is reliable only in RF-negative samples. None of the RF removal methods significantly improved the results. In RF-positive samples, which represent the vast majority of RA patients, cytokines cannot be measured using this technology.

A79 SYNDECAN-4 DEFICIENCY PREVENTS CARTILAGE DESTRUCTION IN THE ANTIGEN-INDUCED ARTHRITIS MODEL OF RHEUMATOID ARTHRITIS

D Wendelt, 1A Stratis, 1K Neugebauer, 1MA Peters, 1B Dankbar, 1C Wunnau, 1F Echtermeyer, 1T Pap. 1Division of Molecular Medicine of Musculoskeletal Tissue, University Hospital, Muenster, Germany; 2Department of Anaesthesiology, Medical School, Hannover, Germany

The interaction of immune cells with synovial fibroblasts has gained increasing attention in understanding the pathogenesis of rheumatoid arthritis (RA). Activated T cells stimulate monocytes, macrophages and synovial fibroblasts to release cytokines such as IL-1 and matrix metalloproteinases (MMP). Recent data show that the transmembrane heparansulfate proteoglycan syndecan-4 is involved critically in cartilage breakdown in RA by providing essential co-signals for ERK phosphorylation and subsequent MMP production in RA synovial fibroblasts (RASF). Here, we sought to investigate further the relevance of these findings by analysing the interaction of syndecan-4 with IL-1 in vitro and by studying the consequences of syndecan-4 deficiency for cartilage breakdown in the murine antigen-induced arthritis (AIA) model of RA.

For in-vitro analysis, human RASF as well as wild-type murine fibroblasts were stimulated with His-tagged IL-1, and the binding of IL-1 to syndecan-4 was assessed using an Ni-NTA column and Western blot. Reconstitution of syndecan-4 knockout fibroblasts with different mutants of syndecan-4 was performed to analyse the relevance of different domains of syndecan-4 for ERK phosphorylation. Experiments applying the IL-1 receptor antagonist (IL-1Ra) along with IL-1 were undertaken to dissect syndecan-4 and IL-1 receptor pathways. For in-vivo analysis, AIA was induced in wild-type and syndecan-4 knockout mice. On day 42, knee joints of these animals were prepared, embedded in paraffin and subjected to histological analysis. Toluidine blue staining of 4 µm tissue sections of the knee joints were used to determine cartilage destruction. For further investigations, the tissue specimens were immunostained for MMP-3 expression.

Stimulation of human synovial fibroblasts with IL-1 led to strongly increased ERK phosphorylation in vitro. This effect could be reduced either by inhibition of the IL-1 receptor (IL-1R) or by inhibition of syndecan-4 and could almost be abrogated by the simultaneous inhibition of both molecules. Reconstitution of syndecan-4-deficient murine fibroblasts with a full-length syndecan-4 construct caused IL-1-mediated ERK1/2 phosphorylation, while cells expressing syndecan-4 without the cytoplasmic domain showed no ERK1/2 phosphorylation. Histomorphological analysis revealed a significantly reduced loss of proteoglycans and less cartilage destruction in the syndecan-4-deficient mice compared with wild-type animals. Immunostaining of the tissue sections demonstrated a decrease of MMP-3 expression in the synovium of syndecan-4 knockout in comparison with wild-type controls.

Our data demonstrate that syndecan-4 contributes critically to IL-1-dependent ERK phosphorylation in synovial fibroblasts and is
involved in IL-1-mediated MMP expression elevating cartilage destruction in AIA. Syndecan-4 is thus a promising novel target for interfering with matrix degradation in arthritis and potentially other inflammatory conditions.

**A80 SYSTEMIC DELIVERY OF ADENOVIRUS ENCODING SUPPRESSOR OF CYTOKINE SIGNALLING TYPE 3 MARKEDLY AFFECTS CELLULAR IMMUNE RESPONSES AND PREVENTS DEVELOPMENT OF COLLAGEN-INDUCED ARTHRITIS**

1'S Veemenbergen, 'M Bennink, 'J Biton, 'N Hessisse, 'O Amta, 'W van den Berg, 'F van de Loo. 'Rheumatology Research and Advanced Therapeutics, Rheumatology, Radboud University, Nijmegen Medical Centre, Nijmegen, The Netherlands; 'EA-4222, University Paris 13, Rheumatology Department, Avicenne Hospital, AP-HP, Bobigny, France

**Introduction:** Members of the suppressor of cytokine signalling (SOCS) family are key negative intracellular regulators of cytokine and growth factor responses, including those that regulate immune responses. The aim of this study was to determine the immunoregulatory effect of SOCS3 on cellular immune responses in mice developing collagen-induced arthritis (CIA).

**Methods:** DBA/1 mice were immunised with type II collagen and adenosiviral vectors were administered by intravenous injection one day after the booster immunisation, that is before the clinical onset of CIA. Invariant natural killer T (NKT) cells were stained with an alphaGalCer-loaded CD1d tetramer and were, together with the T helper (Th) populations, analysed by flow cytometry. Splenic cellular responses were analysed measuring cytokine production by Luminex multi-analyte technology. Collagen type II-specific cell proliferation was determined by thymidine incorporation assay.

**Results:** Systemic delivery of adenovirus encoding SOCS3 resulted in enhanced transgene expression in both liver and spleen. The induction of CIA caused an increase in liver and spleen invariant NKT cells in control mice, which was markedly reduced in SOCS3-treated mice. In addition, liver CD4+TCRbeta+ T lymphocytes produced lower levels of both IFNγ and IL-4 (intracellular staining) after SOCS3 treatment. In the spleen, especially the antigen-presenting cells (APC) showed high SOCS3 transgene expression, which led to decreased production of IL-23, IL-6 and tumour necrosis factor alpha, but significantly higher levels of anti-inflammatory IL-10 by these cells. FACs analysis showed increased numbers of splenic CD4 T cells after SOCS3 treatment. However, purified splenic CD3+ T cells showed, in the presence of SOCS3-transduced APC, reduced antigen-specific proliferation and a significant reduction in IFNγ (~48%), IL-4 (~41%) and IL-17 (~70%) production. Interestingly, the altered cellular immune responses were accompanied by a protective effect on CIA development and histological analysis of knee joints showed reduced joint inflammation and connective tissue destruction.

**Conclusion:** This study demonstrates effective prevention of CIA after intravenous overexpression of SOCS3 due to immunomodulatory effects on T-cell responses in both liver and spleen.

**A81 TH17 PROFILE IN SYSTEMIC SCLEROSIS DISTINGUISHES DIFFERENT SYSTEMIC SCLEROSIS CLINICAL PHENOTYPES**

1'T Radstrate, 1'V van Bon, 1'J Broen, 1'A Hussiani, 1'R Hesselstrand, 1'D Wuttge, 1'Y Deng, 1'R Simms, 1'E Lubberts, 1'R Lafayatis. 1Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands; 1Boston University School of Medicine, Department of Rheumatology, Boston, USA; 1University of Lund, Department of Rheumatology, Lund, Sweden; 1Erasmus Medical Center, Department of Rheumatology, Rotterdam, The Netherlands

**Background and Objectives:** Systemic sclerosis (SSc) is an autoimmune disease in which great controversy over the role of Th1/Th2 balance dominates. Here we investigated whether the recently discovered Th17 pattern was present and if SSc clinical phenotypes could be stratified using the combination of IL-17 with IFNγ or transforming growth factor beta (TGFβ).

**Methods:** Patients were subdivided into having limited cutaneous SSc (lcSSc, n = 12) or diffuse cutaneous SSc (dcSSc, n = 24). A further subdivision was made between early dcSSc (n = 11) and late dcSSc (n = 13) based on the duration of disease, defining early dcSSc as patients having a disease duration of less than 2 years and late dcSSc as patients having a disease duration longer than 3 years. As a comparator group 14 healthy controls were studied. CD5+ cells were isolated using FACS and subsequently studied for the expression of CD4, CD8, CD25, CD45R0, CD45Ra, IL-23, GITR, TGFβ, and intracellular expression of IL-17, TGFβ and IFNγ using flow cytometry. Levels of IL-17, IL-6, IL-1α and IL-23 were performed using Bioplex assays.

**Results:** SSc patients have more CD4 cells, which display a more activated phenotype, as reflected by their increased expression of CD69 and GITR. In addition, CD4, CD45R0 and CD45Ra cells from SSc patients highly express the IL23 receptor. Interestingly, CD45R0 and CD45Ra cells from all SSc patients express high levels of IL-17, whereas IFNγ and TGFβ were selectively upregulated on T cells from patients with lcSSc/edSSc and lcSSc/idSSc, respectively. In line with these observations, T cells isolated from these SSc phenotypes spontaneously secrete higher levels of these cytokines. In line with these observations, circulating levels of IL-17-inducing cytokines IL-6, IL-23 and IL-1α were increased in all or a particular subset of SSc patients. In contrast, IL-17 itself was not detected in the majority of SSc patients.

**Discussion:** The combination of IL-17, IFNγ and TGFβ levels in CD45R0 and CD45Ra cells from SSc patients is useful to distinguish between lcSSc, IdSSc or edSSc. Blocking Th17 inducing cytokines such as IL-6 and IL-23 may provide a useful tool to intervene in the progress of SSc.

**A82 THE ANGIOPOIETIN 1 AND 2 RECEPTOR TIE2 IS ACTIVATED IN RHEUMATOID ARTHRITIS SYNOVIAL MACROPHAGES AND MEDIATES NUCLEAR FACTOR KAPPA B-DEPENDENT IL-6 PRODUCTION**

S Krausz, D de Launay, M Holshierme, C Ambaras, DM Gerlak, D Baeten, PP Tak, K Reedquist. Division of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

**Background and Objectives:** Angiogenesis makes critical contributions to inflammation and joint destruction in rheumatoid arthritis (RA). Angiopoietins (Ang)-1 and 2, which mediate blood vessel remodelling, as well as their receptor, Tie2, are expressed in RA synovial tissue. Although Tie2 expression has been reported on synovial endothelial cells, fibroblast-like synoviocytes (FLS) and macrophages, little is known about how Tie2 signalling contributes to pathology in RA. Here, we examined the cellular distribution of active, phosphorylated active Tie2 in RA synovial tissue.

**Methods:** Double immunofluorescence was performed on RA synovial tissue sections, using anti-phospho (p)-Tie2 antibodies in combination with antibodies recognising cellular markers (CD3, CD22, CD31, CD55, CD68, CD163 and von Willebrand factor) or the activated mitogen-activated protein kinase p-ERK, p-JNK and p-p38. Human peripheral blood (PB) monocytes and PB-derived macrophages were examined for Tie2 expression by FACS analysis. Macrophage IL-6 and IL-8 production was assessed by ELISA after stimulation with medium, Ang-1 or Ang-2, alone or in combination with tumour necrosis factor alpha (TNFα). Ang-1 and Ang-2-dependent activation of Tie2, p38, ERK, JNK, protein kinase B and nuclear factor kappa B (NF-kB) signalling pathways in macrophages was monitored by immunoblotting, and we assessed the influence of inhibitors of these pathways on Ang-induced macrophage cytokine production.

**Results:** Immunofluorescence revealed active Tie2 predominantly in synovial macrophages and only infrequently in endothelial cells and FLS. Low levels of Tie2 expression were observed on PB monocytes and PB-derived macrophages. Macrophage Tie2 was
functional, as stimulation with Ang-1, and to a lesser extent Ang-2, induced phosphorylation of p38, ERK, protein kinase B and inhibitor of nuclear factor kappa isoform alpha in macrophages. In RA synovial macrophages, active Tie2 co-localised with cells expressing phosphorylated ERK, but not p38 or JNK. Ang-1, but not Ang-2, stimulated IL-6 production in macrophages, while not influencing IL-8 production. Ang-1 also cooperated with TNFα in stimulating IL-6 production. Tie2-mediated IL-6 production was significantly blocked by pharmacological inhibition of NF-κB signalling.

Conclusions: Macrophages are the primary targets of Ang/Tie2 signalling in RA synovial tissue. Tie2 activation results in the activation of multiple signalling cascades in macrophages, of which NF-κB is required for IL-6 production. Our results suggest a novel mechanism by which macrophages contribute to angiogenesis in active RA.

THE EFFECTS OF GLUCOCORTICOID ON NUCLEAR FACTOR KAPPA B ACTIVATION IN PERIPHERAL BLOOD MONONUCLEAR CELLS IN PATIENTS WITH SYSTEMIC RHEUMATIC DISEASES

J Swierkot, R Miedzybrodski, B Bukaś, M Młotkiewicz, E Zaczynska, A Czarny, W Gorczyca, J Szechinski. Department of Rheumatology, Medical University, Wrocław, Poland; Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Background: Glucocorticoids have been successfully used in the treatment of rheumatic diseases. They exert their immunosuppressive and anti-inflammatory action mainly on monocytes/macrophages and lymphocytes. It was shown that they may also influence the activity of nuclear factor kappa B (NF-κB) in different cells.

Objective: The aim of this work was to determine the influence of the glucocorticoids on the activity of NF-κB in peripheral blood mononuclear cells (PBMC) in patients with systemic rheumatic diseases.

Materials and Methods: The DNA-binding activity of NF-κB was observed using electrophoretic mobility shift assay (EMSA). Nuclear protein expression was observed using immunohistochemistry. The observations were carried out in patients with rheumatoid arthritis, lupus erythematous, mixed connective tissue disease, Still disease, polymyalgia rheumatica, dermatomyositis, Wegener granulomatosis and healthy volunteers. PBMC were isolated from patients’ blood before oral glucocorticoid therapy, 3–7 days after, and for patients on stable doses of oral glucocorticoids. Moreover, patients presenting with an inadequate response to oral glucocorticoids subjected to the glucocorticoid pulse were studied before and up to 48 h after the pulse. Clinical evaluation for disease activity included physicians’ global assessments, erythrocyte sedimentation rate and serum C-reactive protein level.

Results: EMSA showed increased NF-κB DNA-binding activity in nuclear extracts from PBMC of patients on a stable oral dose of glucocorticoids (5–30 mg prednisolone, n = 16) when compared with the control group (n = 12). Also the percentage of PBMC expressing the active form of NF-κB was higher in glucocorticoid-treated patients than in controls. In patients who began oral glucocorticoid therapy (n = 7) we observed a tendency to decrease NF-κB DNA-binding activity in nuclear PBMC extracts caused by oral glucocorticoids, which was accompanied by a good clinical response. Interestingly, in patients receiving pulses of methylprednisolone (n = 22), we failed to observe any changes in NF-κB DNA-binding activity in PBMC despite the good clinical response to intravenous glucocorticoids. However, a significant decrease in PBMC expressing NF-κB was shown.

Conclusion: Our results suggest that oral glucocorticoid therapy may decrease the activity of NF-κB in PBMC of patients with inflammatory rheumatic disorders. Intravenous glucocorticoid administration in high doses (pulses) may have a different influence on NF-κB in inflammatory cells and cause positive clinical effects in patients by mechanisms other than those of oral glucocorticoids.

THE OSTEOPROTEGERIN/TUMOUR NECROSIS FACTOR-RELATED APOTOPISIS-INDUCING LIGAND RATIO: A PREDICTIVE FACTOR OF REMISSION IN RHEUMATOID ARTHRITIS

1R Audo, C Lukas, M Hahne, B Combe, J Morel. 2IMM, CNRS UMR 5535, Montpellier, France; 2Department of Immunorheumatology, CHU Lapeyronie, Montpellier, France

Introduction: The tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) ratio was shown in several animal models of inflammatory arthritis as a protective factor, suggesting that it could be used as a potential treatment in rheumatoid arthritis (RA). TRAIL can bind to different receptors including osteoprotegerin (OPG), which is also known as a receptor of RANKL. Because the role of TRAIL in patients with RA is not well established, we measured serum levels of TRAIL and OPG in patients with RA.

Patients and Methods: Levels of TRAIL and OPG in serum were measured by ELISA in 68 RA patients who fulfilled the American College of Rheumatology criteria, recent (<2 years), active (>3 swollen joints) not treated or with a stable background treatment for at least 1 month (M0). 33 of the RA patients were followed up at 6 months (M6). 58 osteoarthritic patients were included as controls. Means between groups of patients were compared using the Mann–Whitney test. For comparison of values variation between M0 and M6, the Wilcoxon test was used.

Results: At baseline, TRAIL tended to be higher in RA patients compared with osteoarthritis (OA) patients (1.44 ± 0.14 ng/ml and 1.17 ± 0.07 ng/ml; p = 0.053) and OPG is significantly higher in the RA patients (2.16 ± 0.79 ng/ml and 1.82 ± 0.53 ng/ml; p = 0.02). For the 33 RA patients with partial remission seen after 6 months, disease activity score (DAS28) decreased significantly (p<0.0001). TRAIL, OPG and the ratio OPG/TRAIL were also significantly decreased (p<0.001 for TRAIL and OPG; p = 0.02 for the ratio OPG/TRAIL). Furthermore, we observed that for patients in remission (DAS28 <2.6) at M6 (n = 13), TRAIL at baseline tends to be higher than in patients without remission at 6 months (n = 20) (1.74 ± 1.57 and 1.11 ± 1.02, respectively; p = 0.06) and the OPG/TRAIL ratio is significantly lower in patients in remission than in the others (1.46 ± 0.72 and 2.39 ± 2.36; p = 0.044).

Conclusion: Concentrations of TRAIL and OPG significantly decreased between M0 and M6 and reached those found in OA patients. A low OPG/TRAIL ratio at baseline was associated with remission evaluated on DAS28 at 6 months. The OPG/TRAIL ratio could be used as a predictive factor for remission in RA, but this hypothesis has to be confirmed in a larger cohort.

THE TYPE I INTERFERON SIGNATURE DETERMINES THE SUSTAINED ANTI-CITRULLINATED PROTEIN ANTIBODY LEVELS DURING TUMOUR NECROSIS FACTOR ALPHA BLOCKADE IN RHEUMATOID ARTHRITIS

1T Cantaert, L van Baarsen, C Wijbrands, M van de Sande, J van der Poow Kraan, C Verweij, P P Tak, O Baeten. Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; 2Department of Pathology, VU Medical Center, Amsterdam, The Netherlands; Molecular Cell Biology and Immunology, VU Medical Center, Amsterdam, The Netherlands

Objective: We recently described a type I interferon (IFN) signature in rheumatoid arthritis (RA). The relationship with autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) is unknown. As these antibodies
are differentially regulated during tumour necrosis factor (TNF) blockade in vivo and as TNFζ neutralisation sustains IFNγ production in vitro, we investigated the role of type I IFN in the regulation of autoantibody levels during TNF blockade and in ACPA-positive individuals without arthritis.

Patients and Methods: Serum and peripheral blood mononuclear cells were obtained from 21 RA patients at weeks 0 and 24 of infliximab treatment, from eight ACPA-positive individuals without arthritis and from 10 ACPA-negative healthy controls. The type I IFN signature was determined by peripheral blood cell gene expression analysis. ACPA IgG and IgM, RF IgM, anti-nucleosome IgM and anti-dsDNA were measured by ELISA.

Results: Nine RA patients had a type I IFNlow signature and 12 RA patients had a type I IFNhigh signature. This signature was not related to the presence and levels of ACPA and RF during active disease. TNF blockade by infliximab induced a similar clinical response, a similar induction of antinuclear antibodies and a similar decrease in RF titres in both type I IFNlow and IFNhigh patients. However, ACPA IgG levels were exclusively downmodulated in the type I IFNlow group (611 U/ml to 344 U/ml; p = 0.023) but remained stable in the type I IFNhigh group. This was confirmed by a decrease of ACPA IgM in the type I IFNlow group (7.4 relative units/ml to 5.1 relative units/ml; p = 0.008) but not in the type I IFNhigh group. In contrast to the situation after TNF blockade, the early phase of the ACPA response before the onset of inflammation and disease was not related to an increase in type I IFN. The changes in ACPA IgM and IgG were not related to changes in the disease activity score.

Conclusions: Type I IFN do not determine the humoral autoimmune response in RA, with the exception of the persistence of ACPA levels after TNF blockade.

Results: CZP, adalimumab and infliximab significantly reduced the antibody-binding capacity (×10^4 per cell) of TLR2 versus controls to 13.1 (vs 28.5 for Fab' PEG reagent), 14.3 and 18.1 (vs 29.1 for IgG1 control), respectively; TLR4 binding was reduced to 2.7 (vs 5.7), 2.8 and 2.5 (vs 5.7), respectively; and CD14 to 126 (vs 173), 124 and 136 (vs 171), respectively (Bonferroni multiple comparison test). Etanercept reduced TLR2 and TLR4 levels less than the other anti-TNF to 24.1 and 5.1, respectively (not significant vs control), whereas CD14 was similarly reduced to 129.5. In addition, etanercept affected the levels of TLR2 and TLR4 mRNA to a much lesser degree than the other three anti-TNF.

Conclusions: CZP, adalimumab and infliximab all dramatically reduced the cell surface protein and mRNA levels of TLR2 and TLR4 in lipopolysaccharide-activated cells. The lower levels of these surface markers may explain why monocytes are unresponsive to lipopolysaccharide following CZP, adalimumab and infliximab (due to the reduced capacity of these cells to bind lipopolysaccharide and transmit the inflammatory signal). Etanercept did not reduce the levels of TLR2 and TLR4 to the same degree and does not potently inhibit lipopolysaccharide-driven cytokine production.
**A88** VITAMIN D AND IL-27, BUT NOT IL-10, ARE STRONG INHIBITORS OF TH17 POLARISATION USING DIFFERENT MECHANISMS

1AM Mus, 1JP van Hamburg, 1P Asnawiđija, 2M Hazes, 2R Dolhain, 2E Colin, 2E Lubberts, 1Erasmus Medical Center, Departments of Rheumatology and Immunology, Rotterdam, The Netherlands; 2Erasmus Medical Center, Department of Rheumatology, Rotterdam, The Netherlands

**Introduction:** Recently, we showed that vitamin D inhibits human IL-17 production and stimulates IL-4 production by peripheral blood mononuclear cells from early rheumatoid arthritis (RA) patients. Furthermore, it has been shown that vitamin D inhibited collagen-induced arthritis (CIA) and diminished the severity of existing CIA, although the mechanism is unknown.

**Objective:** To identify the effect of vitamin D on Th1 and Th17 polarisation ex vivo in the prone autoimmune DBA-1 mice with and without CIA and to evaluate overlap or distinction in the mechanism of action of vitamin D with IL-27 and IL-10.

**Methods:** Splenic CD4 T cells of naive DBA-1 and type II collagen immunised DBA-1 mice were isolated using the MACS system. These cells were stimulated in vitro under Th1 (IL-12/anti-IL-4 antibody) or Th17 (IL-23, transforming growth factor beta (TGFβ)) and IL-6 with anti-IL-4 and anti-IFNγ antibodies) conditions with or without vitamin D, IL-10, and IL-27. Intracellular stainings for IL-17A (Th17), IFNγ (Th1), IL-4 (Th2), Foxp3 (T-regulatory cells), IL-2 and IL-10 were evaluated by FACS analysis. In addition, cytokine-specific ELISA were performed to measure protein levels of IL-2 and IL-10 were evaluated by FACS analysis. In addition, cytokine-specific ELISA were performed to measure protein levels of IL-2, IL-10, IL-17, IFNγ, IL-4, IL-6 in the culture supernatant. Moreover, messenger RNA expression of STAT-1, STAT-3, STAT-4, SOCS3, T-bet, GATA-3, and RORγt was measured using qPCR.

**Results:** A high percentage of IL-17 plus IFNγ (Th17) cells were found under Th17 polarising conditions in naive and even higher in CIA-immunised DBA-1 mice. Vitamin D and IL-27 significantly inhibited Th17 polarisation in both spleen cells from naive and CIA-immunised DBA-1 mice accompanied by an increase in IFNγ-producing cells. In contrast, both low and high levels of IL-10 did not inhibit Th17 differentiation. Vitamin D showed an increase in IL-4 and IL-10-positive cells under Th17 polarising conditions of both naive and CIA-immunised DBA-1 mice. This was accompanied by an increase in IL-4 levels in culture supernatant by vitamin D but not by IL-27. Interestingly, double positive cells for IL-17/IL-10 were induced by vitamin D under Th17 conditions of both naive and CIA-immunised mice. In contrast, IL-27 shows this phenomenon only in splenic T cells from CIA-immunised DBA-1 mice.

**Conclusion:** These data show that both vitamin D and IL-27, but not IL-10, are strong inhibitors of Th17 polarisation of splenic T cells from naive and CIA-immunised DBA-1 mice. In contrast to IL-27, vitamin D increases IL-4 levels under Th17 polarising conditions. Furthermore, vitamin D is a strong inducer of IL-17/IL-10 double positive cells under Th17 conditions. These data show differences in the mechanism of IL-27 and vitamin D in the inhibition of Th17 differentiation. Furthermore, it suggests that vitamin D in the presence of TGFβ/IL-6 and IL-23 promotes the generation of the anti-inflammatory IL-17/IL-10 double positive T cells.

**A89** DISTRIBUTION OF INFLAMMATORY CELLS AT THE AUTOIMMUNE MINOR SALIVARY GLAND LESIONS OF SJÖGREN’S SYNDROME: CORRELATION WITH ADVERSE PROGNOSTIC FACTORS

1MI Christodoulou, 1EK Kapsogeorgou, 1NM Moutsopoulos, 1HM Moutsopoulos, 2Pathophysiology Department, School of Medicine, National University of Athens, Athens, Greece; 2Oral Infection and Immunity Branch, National Institute of Dental and Craniofacial Research, NIH, Bethesda, Maryland, USA

Sjögren’s syndrome (SS) is an autoimmune exocrinopathy (mainly of the salivary and lacrimal glands) associated with glandular lymphocytic infiltrates that extend from mild to severe lesions. The infiltrates mainly consist of T and B cells, whereas classic antigen-presenting cells are primarily reported in heavy infiltrates. Nevertheless, variations in the cellular composition of SS inflammatory lesions of distinct severity are not well defined.

**Purpose:** To study the distribution of the various types of infiltrating mononuclear cells (MNC) at SS autoimmune minor salivary gland (MSG) lesions of variable degree and possible correlations with adverse prognostic factors.

**Methods:** MSG biopsy specimens from 26 SS patients with mild (n = 9), intermediate (n = 8) or severe (n = 9) MSG lesions (Tarpley score 1+, 2+ or 3+/4+, respectively) were studied. T cells, T-regulatory cells (Treg), B cells, macrophages (MΦ), interdigitating and follicular dendritic cells (DC) were identified immunohistochemically by antibodies to CD3, FOXP3, CD20, CD68, S100 and fascin, respectively. Positively stained cells were counted field by field in each section and expressed as incidence (percentage of total infiltrating MNC). Statistical analyses were performed by Mann-Whitney or Spearman tests.

**Results:** T cells were found to predominate in mild lesions, whereas B cells predominated in severe lesions (see table). CD68+ MΦ incidence was positively and S100+ DC incidence was negatively associated with the grade of inflammation (see table). Compared with mild or severe infiltrates, higher FOXP3+ Treg incidence was detected in intermediate lesions (see table). T-cell and S100+ DC incidence was negatively (r = −0.20, p = 0.002) and positively (r = 0.54, p = 0.005, respectively), whereas B-cell and CD68+ MΦ incidence was positively correlated (r = 0.79, p < 0.0001 and r = 0.43, p = 0.03, respectively) with biopsy focus score. The presence of salivary gland enlargement was found to correlate with lower T cell (29.8 ± 5.2 vs 49.6 ± 5.2, p = 0.02 in patients with vs patients without) and S100+ DC incidence (0.29 ± 0.05 vs 0.77 ± 0.15, p = 0.04), as well as with higher B-cell incidence (64.5 ± 4.8 vs 44.4 ± 5.3, p = 0.009). Treg incidence correlated with serum C4 levels (r = 0.54, p = 0.015).

**Conclusion:** The distribution of the infiltrating MNC populations at the MSG lesions of SS varies according to the grade of inflammation and correlates with certain adverse prognostic factors. The significance of this differential distribution of lymphocytic cell populations, as well as of the mediating aetiopathogenic factors, needs to be elucidated.

Abs 89 Table Incidence of the infiltrating inflammatory cell types at the SS autoimmune MSG lesions (incidence: percentage of total infiltrating mononuclear cells)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Mild</th>
<th>Intermediate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ T cells</td>
<td>64.05 ± 4.60</td>
<td>36.21 ± 2.61</td>
<td>25.42 ± 3.35</td>
</tr>
<tr>
<td>FOXP3+ Treg</td>
<td>1.56 ± 0.35</td>
<td>2.29 ± 0.31</td>
<td>1.08 ± 0.16</td>
</tr>
<tr>
<td>CD68+ B cells</td>
<td>30.87 ± 4.63</td>
<td>57.87 ± 3.66</td>
<td>67.12 ± 3.33</td>
</tr>
<tr>
<td>CD68+ macrophages</td>
<td>1.93 ± 0.85</td>
<td>3.36 ± 1.10</td>
<td>5.50 ± 1.04</td>
</tr>
<tr>
<td>S100+ interdigitating DC</td>
<td>0.92 ± 0.19</td>
<td>0.56 ± 0.17</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>Fas+ follicular DC</td>
<td>1.58 ± 0.35</td>
<td>1.68 ± 0.37</td>
<td>1.45 ± 0.50</td>
</tr>
</tbody>
</table>

Mean percentage ± SE. DC, dendritic cell; MSG, minor salivary gland; Treg, T-regulatory cell.
6. Innate immunity, infection and inflammation

A90 12/15-LIPOXYGENASE COUNTERACTS INFLAMMATION AND TISSUE DAMAGE IN ARTHRITIS

1G Krönke, 1J Katzenbeisser, 1U Uderhardt, 1M Zaiss, 1G Schabauer, 1A Zarbock, 3M Koniers, 1R Axmann, 1J Zwerina, 1L Joosten, 1G Schett. 1Department of Internal Medicine 3 and Institute for Clinical Immunology, University of Erlangen-Nuremberg, Erlangen, Germany; 2Department for Vascular Biology, Center for Biomolecular Medicine and Pharmacology, Medical University Vienna, Vienna, Austria; 3Department of Anesthesiology and Intensive Care Medicine, University of Münster, Münster, Germany; 4Department of Rheumatology and Department of Medicine, Radboud University, Nijmegen Medical Centre, Nijmegen, The Netherlands

Background and Objectives: Lipoxin A4 (LXA4) is considered a key anti-inflammatory mediator facilitating the resolution of inflammation, a process essential for protection from overwhelming inflammatory responses and inflammation-associated tissue damage. Leucocyte-type 12/15-lipoxygenase (12/15-LO) represents a major enzyme involved in LXA4 generation. Nevertheless, the impact of 12/15-LO on chronic inflammatory diseases such as arthritis has remained elusive. We therefore sought to determine the role of this enzyme in murine experimental arthritis.

Materials and Methods: We investigated the role of 12/15-LO by performing two experimental models of arthritis, the K/BxN serum-transfer and a tumour necrosis factor (TNF) transgenic mouse model, in wild-type and 12/15-LO-deficient C57/B6 mice.

Results: We showed that the deletion of 12/15-LO led to uncontrolled inflammation and tissue damage in both models of arthritis. 12/15-LO-deficient mice showed an enhanced inflammatory gene expression and decreased levels of LXA4 within their inflamed synovia. Consistent with this, in isolated macrophages, the addition of LXA4 blocked both the phosphorylation of p38MAPK and the expression of a subset of pro-inflammatory genes. Conversely, 12/15-deficient macrophages displayed significantly reduced levels of LXA4, which correlated with increased activation of p38MAPK and an enhanced inflammatory gene expression after stimulation with TNF.

Conclusions: Taken together these results support an anti-inflammatory and tissue-protective role of 12/15-LO and its products during chronic inflammatory disorders such as arthritis and provide novel insights into feedback loops limiting inflammation-associated tissue damage.

A91 ANTI-CITRULLINATED PROTEIN ANTIBODIES NOT ONLY ACTIVATE THE CLASSIC BUT ALSO THE ALTERNATIVE COMPLEMENT PATHWAY

1L Trouw, 1T Haisma, 1N Levahrt, 1D van der Woude, 1A Ioan-Facsinay, 1T Huizinga, 1R Toes. 1Leiden University Medical Center, Department of Rheumatology, Leiden, The Netherlands

Background and Objectives: Recent studies in mice show that arthritis-inducing antibodies do not require the classic pathway of complement activation, the pathway traditionally associated with antibody-mediated defences. Surprisingly, they employ the alternative pathway. In humans anti-citrullinated protein antibodies (ACPA) are prime suspects in the pathogenesis of rheumatoid arthritis (RA). Here we have analysed which pathways of complement are activated by ACPA.

Materials and Methods: We have set up assays to measure complement activation by these antibodies by using cyclic citrullinated peptide coated plates (CCP2), specific buffers that allow the binding of ACPA without inducing complement activation during the binding step, followed by complement activation from a pool of normal human serum. Specific activation of different complement pathways was achieved by using specific buffers and deficient sera. ACPA isotype usage was tested in relation to complement activation.

Results: We observed that ACPA activate complement in a dose-dependent manner via the classic and intriguingly also strongly via the alternative pathway, mimicking the situation in mice. We did not observe any lectin pathway activation, also not in those patients positive for IgA ACPA. Complement activation proceeded in vitro up to the formation of the membrane attack complex C5b9, showing that all activation steps, including the release of C5a, have been taken. The extent of complement activation is proportional to the ACPA titre.

Conclusions: These data show that ACPA not only activate the classic pathway but also strongly activate the alternative pathway. These findings provide a rationale to target the alternative pathway to inhibit disease progression in RA patients.

A92 ANTI-OXIDISED LOW-DENSITY LIPOPROTEIN ANTIBODIES AND ANTI-OXIDISED LOW-DENSITY LIPOPROTEIN BETÀ GLYCOPROTEIN I ANTIBODIES IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

1B Nowak, 1M Szmyrka-Kaczmarek, 1A Durazinska, 1L Korman, 1P Plaskej, 1P Wlond. 1Department of Pharmacology, Silesian Piast University of Medicine, Wrocław, Poland; 2Department of Rheumatology and Internal Diseases, Silesian Piast University of Medicine, Wrocław, Poland; 3Department of Cardiology, Silesian Piast University of Medicine, Wrocław, Poland

Background and Objectives: As the premature development of atherosclerosis in patients with systemic lupus erythematosus (SLE) cannot be fully explained by traditional risk factors or treatment complications we planned to assess the concentration of anti-oxidised low-density lipoprotein (oxLDL) antibodies and antibodies to oxLDL β2 glycoprotein I (β2 GPI) complexes in the sera of SLE patients. Atherosclerosis is characterised by a gradual thickening of arterial walls caused by the accumulation of lipids. Experimental evidence indicates that pro-inflammatory factors and dyslipidaemia are the main contributors to the development of atherosclerotic plaques. Low-density lipoprotein (LDL) is the main form of cholesterol that accumulates in those lesions. Before it is inserted into the plaque LDL must be modified into an oxidised structure (oxLDL). Unlike LDL, oxLDL binds to β2 GPI and forms the oxLDL–β2 GPI complex, which has been implicated as a pro-atherogenic agent. An active macrophage uptake of immune complexes consisting of oxLDL β2 GPI antibodies leads to the accelerated development of foam cells and atherosclerotic plaque.

Materials and Methods: 16 SLE patients (14 women, two men) and 13 healthy volunteers (13 women) were enrolled in the study. Anti-oxLDL antibodies and anti-oxLDL β2 GPI antibodies (IgM and IgG) levels were assessed in the sera using ELISA tests. Disease activity was measured using the SLEDAI index.

Results: Anti-oxLDL antibody concentration was significantly higher in the sera of SLE patients than in the control group (97.83 ± 22.58 µl/l vs 70.24 ± 18.98 µl/l, p = 0.0016, respectively). We also detected a statistically significant difference between the levels of IgM anti-oxLDL β2 GPI antibodies (38.67 ± 40.96 U in the SLE group vs 11.93 ± 5.57 U in the control group, p = 0.028). The difference in IgM anti-oxLDL β2 GPI antibody concentration between both groups was statistically insignificant, although there was a tendency to higher levels in the SLE group (42.06 ± 63.65 U vs 11.69 ± 6.74 U, p = 0.099). The concentrations of IgM anti-oxLDL β2 GPI antibodies and IgG anti-oxLDL β2 GPI antibodies were not correlated with each other. In the SLE group statistically significant correlations between anti-oxLDL concentration and LDL, triglyceride and total cholesterol levels were found (relative proportion (RP) 0.68, RP 0.61 and RP 0.51; p<0.05, respectively). In the SLE group no correlation between disease activity measured with the SLEDAI and oxLDL, IgM anti-oxLDL β2 GPI antibodies and IgG anti-oxLDL β2 GPI antibodies was found. However, there was a statistically significant negative correlation between C-reactive protein and the high-density lipoprotein concentration (RP −0.60, p<0.05).
Conclusion: The results of our study confirm previously described correlations between lupus dyslipoproteinaemia and autoantibody synthesis and the inflammatory process in SLE. These two processes seem to be implicated in premature atherosclerosis, important causes of morbidity reported in SLE patients.

A93 BIOLOGICAL ROLE OF HEPATOCYTE-DERIVED IL-1 RECEPTOR ANTAGONIST IN A MODEL OF SYSTEMIC INFLAMMATION

C Lamacchia, G Palmer, D Talabat-Ayer, C Gabay. Department of Pathology and Immunology, University of Geneva School of Medicine (CMU), Geneva, Switzerland

Background: IL-1 receptor antagonist (IL-1Ra) is a specific IL-1 inhibitor that possesses anti-inflammatory activities in experimental models and in patients. Hepatocytes produce IL-1Ra in large amounts in response to inflammatory stimuli as an acute-phase protein and were thus suggested to represent a major source of circulating IL-1Ra during systemic inflammation. In addition, IL-1Ra-deficient mice (IL-1Ra KO) have an increased susceptibility to lipopolysaccharide-induced death.

Objectives: The aims of this study were to determine the contribution of hepatocytes as a cellular source of circulating IL-1Ra in response to systemic inflammation induced by lipopolysaccharide injection and also to define the functional role of hepatocyte-derived IL-1Ra in the control of lipopolysaccharide-induced lethality.

Methods: Conditional hepatocyte-specific IL-1Ra-deficient mice (IL-1Ra deltaH) were generated in a pure C57BL/6 genetic background by using the LoxP/Cre-recombinase system. Lipopolysaccharide (2 mg/kg or 10 mg/kg) was injected intraperitoneally into IL-1Ra deltaH and wild-type mice to induce a systemic inflammatory response and IL-1Ra was quantified by ELISA in liver extracts and sera 4 h or 18 h after injection. Lipopolysaccharide (10 mg/kg) was injected intraperitoneally into IL-1Ra deltaH mice, wild-type mice, and IL-1Ra KO mice to assess the survival in these three lines of mice.

Results: After lipopolysaccharide challenge, IL-1Ra messenger RNA and protein levels were specifically decreased by 80% in the livers of IL-1Ra deltaH compared with wild-type mice. Surprisingly, the plasma levels of IL-1Ra were decreased only by 30% in IL-1Ra deltaH compared with wild-type mice, 4 h after the injection of 2 mg/kg lipopolysaccharide. No significant difference was observed 18 h after injection. After injection of 10 mg/kg lipopolysaccharide, the levels of circulating IL-1Ra were decreased by 50% and 66%, respectively, after 4 h and 18 h, in IL-1Ra deltaH compared with wild-type mice. Finally, as opposed to IL-1Ra KO mice, which were highly susceptible to the lethal effects of lipopolysaccharide, there was no difference in survival between control mice and IL-1Ra deltaH mice.

Conclusions: Hepatocytes can be considered a major source of IL-1Ra in the liver in response to lipopolysaccharide. In addition, the results indicate that the contribution of hepatocytes as a source of circulating IL-1Ra in response to lipopolysaccharide is dose dependent and only partial. Finally, hepatocyte-derived IL-1Ra is not required for survival to endotoxinaemia. These observations suggest that other sources of IL-1Ra contribute to circulating levels of IL-1Ra and to survival in this model of sepsis.

A94 DISTINCT TEMPORAL EVOLUTION OF TOLL-LIKE RECEPTOR-MEDIATED DENDRITIC CELL CYTOKINE SECRETION IN PATIENTS WITH LIMITED AND DIFFUSE CUTANEOUS SYSTEMIC SCLEROSIS

1 L van Bon, 2C Papa, 1R Huijben, 3M Vonk, 3M York, 3R Simms, 3R Hesselstrand, 3D Wittge, 2R Lafaytis, 1T Radstake. 1Radboud University, Nijmegen Medical Center, Nijmegen, The Netherlands; 2Bost University School of Medicine, Department of Rheumatology, Boston, USA; 3University of Lund, Department of Rheumatology, Lund, Sweden

Background and Objectives: Systemic sclerosis (SSc) is an autoimmune disease and accumulating evidence suggests a role for Toll-like receptor (TLR)-mediated activation of dendritic cells (DC). Here we mapped TLR-mediated cytokine responses of DC from SSC patients.

Methods: 45 SSc patients were included. Patients were stratified as having diffuse cutaneous SSc (dcSSc) or limited cutaneous SSc (lcSSc) on the basis of the extent of skin involvement and further divided into those having late (lcSSc >3 years, dcSSc >2 years) or early disease. DC were stimulated with ligands for TLR2, TLR3, TLR4, TLR7/8 or combinations. Plasma samples were collected from SSc patients (n = 167) and measured for IL-6, tumour necrosis factor alpha (TNFα), IL-12, IL-10 and IFNy.

Results: Stimulation of DC subsets from early lcSSc and dcSSc patients with ligands for TLR2, TLR3 or TLR4 resulted in greater secretion of IL-6 and TNFα compared with those having late disease or healthy controls. Remarkably, the production of IL-12 was lower upon stimulation with TLR ligands in most SSc patients, whereas the secretion of IL-10 was very high in patients with the dcSSc phenotype, particularly in those having early dcSSc. The combination of various TLR ligands led to reduced cytokine secretion in all SSc patients. Circulating levels of these cytokines further underscored the presence of differences between various SSc phenotypes.

Discussion: The altered TLR-mediated activation of DC may be responsible for the skewed T-cell activation in SSc that may be the cause of fibrogenic T-cell cytokines, such as IL-4 and IL-13. DC targeting could thus offer new avenues for therapeutic intervention.

A95 EFFECTS OF INFliximab ON HAEMOSTATIC AND INFLAMMATORY BIOMARKERS IN PATIENTS WITH ACTIVE RHEUMATOID ARTHRITIS

1F Ingegno, 1F Fantini, 1A Soldi, 2V Galiati, 3S Griffin, 1E Giulio Favalli, 3M Cugno. 1Department of Rheumatology, University of Milan, Istituto Gaetano Pini, Milan, Italy; 2Department of Internal Medicine, University of Milan, IRCCS Fondazione Ospedale Maggiore Policlinico, Milan, Italy

Background and Objectives: 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 (TNFα), reduces inflammation, but its effect on coagulation and fibrinolysis is unknown. We therefore investigated plasma biomarkers of inflammation, coagulation and fibrinolysis before and after infliximab treatment in RA patients.

Materials and Methods: We studied 20 patients with active RA and 40 healthy controls. Patients, long-term treated with a stable dose of methotrexate (10 mg/week), received infliximab (3 mg/kg) at weeks 0, 2, 6 and 14. At baseline and week 14, we determined: disease activity score (DAS28), visual analogue scale pain, erythrocyte sedimentation rate (ESR), and plasma levels of C-reactive protein (CRP), TNFα, IL-6, prothrombin fragment 1+2 (F1+2), D-dimer, plasminogen activator inhibitor type 1 (PAI-1) antigen and PAI-1 activity.

Results: At baseline, plasma levels of both inflammatory (ESR, CRP, TNFα, IL-6) and haemostatic (F1+2, D-dimer, PAI-1 antigen and PAI-1 activity) markers were significantly higher in RA patients than in controls (p = 0.02 to p = 0.0001). At 14 weeks of infliximab treatment, there was a significant clinical improvement and a significant decrease in ESR, CRP, IL-6 (p = 0.02 to p = 0.0001). Furthermore, F1+2, D-dimer, PAI-1 antigen and PAI-1 activity levels decreased significantly (p = 0.01 to p = 0.0001).

Conclusions: In RA patients, infliximab leads to a rapid significant improvement in clinical and laboratory markers of inflammation. Moreover, this study provides evidence that TNFα inhibition with infliximab decreases both the activation of coagulation and the inhibition of fibrinolysis. Such a combined effect may be pivotal in reducing the whole thrombotic risk in these patients.
Background and Objectives: The endogenous protein HMGB1 (high mobility box chromosomal protein type I) is abundantly expressed extracellularly in rheumatoid joint tissue. We have previously reported that therapies targeting HMGB1 significantly improve the disease course of experimental arthritis. New data demonstrate that HMGB1 enhances inflammation by synergising with other molecules such as Toll-like receptor (TLR) ligands and IL-1β. The aim of this study was to determine the effect of stimulation with HMGB1 alone or HMGB1 complexed with low amounts of lipopolysaccharide or IL-1β in rheumatoid arthritis (RA) synovial fibroblasts and in osteoarthritis synovial fibroblasts.

Methods: Synovial fibroblasts obtained from four RA and five osteoarthritis patients were stimulated with HMGB1 alone or with HMGB1 complexed with low amounts of lipopolysaccharide or IL-1β. Tumour necrosis factor (TNF) production was determined after 9 h of stimulation by ELISPOT and IL-6, IL-8, TNF, IL-10 and IL-12 levels were measured after 24 h of stimulation by flow cytometry, using the cytokine bead array (CBA). Matrix metalloproteinase (MMP) type 1 and MMP-3 production was determined by ELISA.

Results: HMGB1 alone did not induce cytokine production as determined by ELISPOT or by CBA. Neither did HMGB1 alone induce MMP-1 or MMP-3 production. In contrast, stimulation with HMGB1 together with trace amounts of lipopolysaccharide or of IL-1β had strong potentiating effects (1–2 log order) on TNF, IL-6 and IL-8 production compared with lipopolysaccharide or IL-1β stimulation alone. The stimulatory capacity was directly dose dependent and the effects of HMGB1–IL-1β complex stimulation could be completely abolished by the addition of soluble IL-1 receptor antagonist (anakinra) to the cell culture. Similar to cytokine production, the production of MMP-1 and MMP-3 was greatly enhanced by stimulation with the investigated HMGB1 complexes.

Conclusions: HMGB1 complexed to lipopolysaccharide or to IL-1β is capable of enhancing proinflammatory cytokine production and MMP production in synovial fibroblasts. We thus suggest that HMGB1 might be an endogenous factor unifying the effects of various microbial-derived substances on RA pathogenesis. By this study, we have extended knowledge regarding the proinflammatory functions of HMGB1 in arthritis, both osteoarthritis and RA. We have demonstrated enhancing effects of HMGB1 on both inflammatory and destructive disease mechanisms and further consolidated HMGB1 as a putative target for successful therapy.
TLR2, TLR4 and CD163 expression. IL-10 strongly upregulated the expression of CD163 and, to a lesser extent, CD16 and TLR4.

**Conclusion:** Our data indicate that CD86, CD14, CD200R and CD163 are specific surface markers for in-vitro polarised M1a, M1b, M2a and M2c macrophages, respectively. In addition, CD206 and CD16 can be useful for the characterisation of M2a and M2c cells. We will evaluate this phenotypic classification in polarised human macrophages in situ and test its correlation with macrophage function and signalling.

**IL-12 AND IL-23 SECRETION BY DENDRITIC CELLS IN SPONDYLOARTHRITIS**

C Prevosto, JC Goodall, JS Hill Gaston. Department of Medicine, University of Cambridge, Cambridge, UK

**Background:** In addition to pathogenic CD4 effector Th1 cells, a new population of IL-17-producing T cells, termed Th17 cells, has recently been described. Th17 cells play a crucial role in autoimmune models, including experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA). Unlike Th1 cells, which require IL-12 for their differentiation, the growth and survival of Th17 cells requires IL-23, a cytokine that combines a p40 subunit shared with IL-12 and a unique p19 subunit; p19-deficient mice are resistant to EAE and CIA. Both IL-12 and IL-23 are produced by dendritic cells (DC); we have measured the secretion of these cytokines by monocyte-derived DC (moDC) from ankylosing spondylitis (AS) patients, compared with moDC from healthy subjects or rheumatoid arthritis (RA) patients.

**Methods:** Monocytes were isolated from peripheral blood of 16 AS and 14 RA patients and eight age-matched healthy subjects, and 25 058 from AS patients (5425 pg/ml, 5670 pg/ml, and 3530 pg/ml, respectively) although these differ-

**Results:** IL-10 strongly upregulated the expression of CD163 and, to a lesser extent, CD16 and TLR4.

**Conclusions:** Together, these findings show that the triggering of TLR3 in combination with TLR7–8 leads to an increase in IL-23 and IL-12 secretion by DC from AS patients compared with healthy donors; however, this finding was not explained by altered DC maturation.

**MODULATION OF MONOCYTE FC GAMMA RIIIA EXPRESSION IN RHEUMATOID ARTHRITIS: EVIDENCE FOR A CHANGE IN PHENOTYPE WITH DISEASE PROGRESSION**

1DL Cooper, 2SL Mackie, 3SG Martin, J Nam, M Buch, 4JO Issacs, 5P Emery, 1AW Morgan. 1Institute of Molecular Medicine, University of Oxford, Oxford, UK; 2Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK

**Introduction:** In human peripheral blood, two monocyte subsets with distinct functional properties have previously been described. These are defined as the CD14+/CD16–/low and a minor subset of CD14low/CD16++ cells. The expression of CD16/Fc gamma R (FcyR) IIIa, an activating FcyR, may make monocytes targets for activation by IgG-containing immune complexes (eg, rheumatoid factor and cyclic citrullinated peptide autoantibodies), which are present in the serum and inflamed joints of rheumatoid arthritis (RA) patients. Our previous data have shown increased levels of CD16/FcRIIIA on the CD14++ monocyte subset in RA compared with healthy controls. This may result in a cell that is more sensitive to immune complex stimulation. We wanted to explore whether upregulated CD16/FcRIIIA expression was associated with a modulation of monocyte function and response to immune complex stimulation. The importance of disease duration, age, level of systemic inflammation and change with therapy with respect to FcRIIIA expression levels was also investigated.

**Methods:** The monocyte phenotype was determined by flow cytometric analysis of whole blood samples, with selective gating on forward scatter and side scatter signals and expression of CD14. The expression of CD16/FcRIIIA on CD14low and CD16++ subset was measured in healthy controls (n = 43) and RA patients (n = 40) at time of initiation of therapy in a randomised controlled trial of infliximab compared with steroid in methotrexate-treated patients to evaluate the modulation of FcRIIIA expression during therapy. The expression of markers for phagocytosis (CD64, CD11b, CD18), antigen presentation (HLA-DR, CD80, CD86), adhesion and migration (intracellular cell adhesion molecule type 1, CXCR1, CCR2) were examined on these monocyte populations to evaluate changes in cellular function.

**Results:** The expression of FcRIIIA/CD16 was increased on CD14++ monocytes in established RA compared with healthy controls (p<0.05). The expression of FcRIIIA/CD16 on CD14++ monocytes in early RA was not significantly different from healthy controls. FcRIIIA/CD16 expression was not correlated with systemic inflammation (C-reactive protein) or age. No significant changes in FcRIIIA/CD16 were seen with infliximab, steroid or methotrexate therapy. The most striking finding from the additional phenotypic analysis on monocyte subsets was increased expression of HLA-DR, CD54, CD86 and CD18 on CD14++/CD16+ cells compared with CD14+/CD16− cells. Studies to evaluate cytokine secretion from monocytes following immune complex stimulation are underway.

**Conclusions:** The increased expression of FcRIIIA on CD14++ monocytes in long-standing RA compared with early RA supports a change in phenotype during disease progression and potential increased sensitivity to immune complexes. These data suggest that this increase in FcRIIIA is not associated with aging or inflammation.

**RHEUMATOID ARTHRITIS-ASSOCIATED AUTOANTIBODIES IN LEISHMANIA DONOVANII INFECTION**

1AI Elshafie, 1EA˚hlin, 3K Pauksens, 1JR o¨nnelid. 1Clinical Immunology Unit, Uppsala University, Uppsala, Sweden; 2Department of Pathology and Microbiology, AIHab University Hospital, Kharoum, Sudan; 3Infectious Diseases Unit, Department of Medical Sciences, Uppsala University, Uppsala, Sweden

**Background and Objectives:** Visceral leishmaniasis (VL) or Kala-azar, is the most severe form of leishmaniasis and is caused by...
parasites of the Leishmania genus. Post-Kala-azar dermal leishmaniasis (PKDL) is a complication of VL characterised by severe rashes and skin lesions in mostly young patients who have recovered from VL. Circulating immune complexes (CIC) are increased in chronic leishmaniasis. Rheumatoid factor (RF) is a common finding in immune complex-associated chronic infectious diseases. In one small study the occurrence of anti-cyclic citrullinated peptide (CCP) antibodies has been described in Brazilian patients infected with Leishmania major. We have investigated the occurrence of different RA-associated autoantibodies in a larger cohort of Sudanese patients infected with Leishmania donovani.

Methods: Serum samples were collected from 82 VL patients (mean age 24 years), 47 PKDL patients (mean age 13 years), 52 healthy Sudanese controls (mean age 14 years) and 54 Sudanese patients seeing a rheumatologist because of joint complaints, either in the Tabarakalla rural area (VL and PKDL) or at Alirbat University Hospital (controls and rheumatology patients). Samples were immediately separated and maintained frozen below −70°C until analyses in Uppsala. Levels of CIC, anti-CCP and anti-human collagen type II antibodies (anti-CII) were investigated by ELISA and RF by nephelometry.

Results: Among VL and PKDL patients 73/83 (mean 225 IU/ml) and 30/42 (mean 47 IU/ml) were RF positive. In patients with joint complaints 18/31 (mean 123 IU/ml) and in the Sudanese control group 26/55 (mean 28 IU/ml) were RF positive. For anti-CCP nine of 80 (11%) VL patients and two of 47 (4%) PKDL patients were positive and seven of 34 (21%) of the joint patients. In the Sudanese control group, one of 52 was positive. The mean levels of anti-CCP among the positive samples (using the Eurodiagnostica kit with a 25E/ml cut-off) were: VL 46 E/ml, PKDL 64 E/ml, joint patients 620 E/ml. Among VL and PKDL patients, 74/82 (90%) and 37/45 (82%), respectively, were positive for anti-CII. Among joint patients 12/34 (35%) had elevated anti-CII levels and in the Sudanese control group 29/52 (56%). In all anti-CII ELISA plates two internal controls failed to reveal a single active TB infection. The cohort received etanercept for a mean of 25.2 months (range 3–60 months), with an overall 168 patient-years of etanercept exposure in PPD-positive individuals. The cohort included 49 Hispanics, 15 African Americans, seven white individuals and seven Asian individuals; 61 (80.2%) were foreign born and 63 (78.7%) had low socioeconomic status. Indications for etanercept use included: rheumatoid arthritis (RA), 56 (70%); ankylosing spondylitis, 10 (12.5%); psoriatic arthritis, 12 (15%); juvenile RA, one (1.25%) and Takayasu’s disease, one (1.25%). 74 of the 80 subjects received full or partial treatment for LTBI before initiating etanercept. LTBI therapy (mostly isoniazid) had been initiated a mean of 2.5 months before etanercept therapy (range 0–12 months). Most patients (72/80) were on one or more immunosuppressive treatments during etanercept therapy.

Conclusions: In this retrospective analysis, we observed no reactivation of LTBI during 168 patient-years of follow-up in 80 patients who were PPD positive and received etanercept. Although small, this systematic analysis of the risk of TB in PPD-positive patients during etanercept therapy suggests that the risk of clinically significant reactivation of LTBI during etanercept therapy must be relatively low in appropriately treated individuals.

A102 SAFETY OF ETANERCEPT IN PATIENTS AT HIGH RISK OF MYCOBACTERIAL TUBERCULOSIS INFECTIONS

1R Aggarwal, 1,2A Manandhar, 1,2A Polyedath, 1,2W Sequeira, 1,2J Block. 1Rush University Medical Center, Chicago, Illinois, USA; 2John Stroger Jr Hospital of Cook County, Chicago, Illinois, USA

Background and Objectives: Etanercept is an anti-tumour necrosis factor alpha (TNFα) agent commonly used to treat a variety of rheumatic diseases. Despite the dramatic clinical benefits associated with its use, there has been concern regarding its association with active tuberculosis (TB). Most TB associated with TNFα agents occurs in patients with positive purified protein derivative (PPD). Here, we evaluated the risk of TB among PPD-positive patients treated with etanercept.

Methods: All patients with a positive PPD, as defined by American Thoracic Society/Centers for Disease Control and Prevention guidelines, who received etanercept from the John H Stroger Jr Hospital of Cook County from January 2001 to October 2007 were retrospectively reviewed. Detailed demographic information, clinical, laboratory and radiological data were obtained by chart review. Patients with positive PPD were considered to have latent TB infection (LTBI). The primary endpoint was the development of active TB either while receiving or after completing etanercept therapy.

Results: 465 patients received etanercept, of which 80 were PPD positive and constituted the primary study cohort. A comprehensive review of all outpatient and inpatient records of these patients failed to reveal a single active TB infection. The cohort received etanercept for a mean of 25.2 months (range 3–60 months), with an overall 168 patient-years of etanercept exposure in PPD-positive individuals. The cohort included 49 Hispanics, 15 African Americans, seven white individuals and seven Asian individuals; 61 (80.2%) were foreign born and 63 (78.7%) had low socio-economic status. Indications for etanercept use included: rheumatoid arthritis (RA), 56 (70%); ankylosing spondylitis, 10 (12.5%); psoriatic arthritis, 12 (15%); juvenile RA, one (1.25%) and Takayasu’s disease, one (1.25%). 74 of the 80 subjects received full or partial treatment for LTBI before initiating etanercept. LTBI therapy (mostly isoniazid) had been initiated a mean of 2.5 months before etanercept therapy (range 0–12 months). Most patients (72/80) were on one or more immunosuppressive treatments during etanercept therapy.

Conclusions: In this retrospective analysis, we observed no reactivation of LTBI during 168 patient-years of follow-up in 80 patients who were PPD positive and received etanercept. Although small, this systematic analysis of the risk of TB in PPD-positive patients during etanercept therapy suggests that the risk of clinically significant reactivation of LTBI during etanercept therapy must be relatively low in appropriately treated individuals.

A103 SYNOVIAL INFILTRATION WITH C-KIT-POSITIVE CELLS IN NON-PSORIATIC AND PSORIATIC SPONDYLOARTHROPATHIES

1T Noordenbos, 1,2JD Cañete, 1T Cantaert, 1N Yerenenko, 1C Teitsma, 1M van de Sande, 1FP Tak, 1D Baeten. 1Clinical Immunology and Rheumatology; Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; 2Arthritis Unit, Rheumatology Department, Hospital Clinic de Barcelona and IDIBAPS, Barcelona, Spain

Objectives: We recently observed a striking synovial infiltration with cells positive for C-kit, a marker for mast cells and haematoipoetic stem cells, in psoriatic arthritis (PsA) (Cañete et al, submitted). As mast cells have potent inflammatory functions, including the production of tumour necrosis factor (TNF), we performed a systematic analysis of C-kit-positive cells in different forms of chronic inflammatory arthritis.

Materials and Methods: Synovial tissue from rheumatoid arthritis (RA) (n = 21), non-psoriatic spondyloarthropathies (SpA) (n = 16) and PsA (n = 23) was stained with toluidine blue for mast cells and analysed by immunohistochemistry for C-kit, tryptase, CD3, CD20 and CD68. Synovial fluid from RA (n = 18), SpA (n = 19) and PsA (n = 16) was analysed by ImmunoCap for tryptase and by ELISA for stem cell factor (SCF), IL-3, IL-5, IL-3 and sST2.

Results: C-kit-positive mononuclear cells were found in the synovial sublining in all disease groups. Semiquantitative scoring showed a significant increase of C-kit-positive cells in SpA (p = 0.010) and PsA (p = 0.001) compared with RA, despite similar levels of global inflammation, as reflected by CD3, CD20 and CD68 staining. Synovial fluid levels of SCF, IL-3 and IL-5, all factors involved in chemotaxis and differentiation of mast cells, as well as sST2, the soluble decoy receptor for IL-33, were similar in all groups. Investigating whether synovial C-kit expression identified infiltrating mast cells, additional stainings with toluidine blue and
antitrypsin showed similar numbers of mast cells in all types of synovitis. Accordingly, synovial fluid trypsin levels were similar in the three disease groups. Double stainings showed that all toluidine blue-positive cells were also C-kit positive, identifying mast cells, but that many infiltrating cells were C-kit single positive.

Conclusion: There is an increased synovial infiltration with C-kit-positive cells in non-psoriatic and psoriatic SpA. As these cells do not appear to be mast cells, we are currently investigating their phenotype and identity.

7. Endothelium and cell recruitment, extracellular matrix and fibroblasts

**A104** CHARACTERISATION OF CD55: A DEFINING MARKER ON FIBROBLAST-LIKE SYNOVIOCYTES

1DP Wijnker, 1PP Tak, 1J Hamann. 1Department of Experimental Immunology, Academic Medical Center, Amsterdam, The Netherlands; 2Division of Clinical Immunology and Rheumatology, Academic Medical Center, Amsterdam, The Netherlands

**Background and Objectives:** CD55 (also known as decay-accelerating factor) is a widely expressed cell-surface molecule that protects self cells from complement-mediated damage by accelerating the decay of C3/C5 convertases. An unusually large amount of CD55 is expressed on fibroblast-like synoviocytes (FLS), a key cellular constituent of the synovial lining layer. CD55 is also a ligand for CD97, a non-classical heptahelical receptor that is broadly expressed on leucocytes. Our group previously demonstrated that CD55 on FLS can interact with CD97 on synovial macrophages. We hypothesise that CD55 on FLS contributes to the retention of inflammatory cells into the synovium in rheumatoid arthritis (RA).

**Materials and Methods:** FLS, grown from synovial tissue biopsies from arthritis patients, were studied between passage 3 and 9. The expression of CD55 was measured by flow cytometry and quantitative real-time PCR. In addition, RA FLS were stimulated with multiple pro-inflammatory cytokines and Toll-like receptor (TLR) ligands and analysed for CD55 expression. The mechanism of poly(I:C) stimulation was studied by blocking the TLR3 pathway with hydroxychloroquin. Binding of multivalent fluorescent probes loaded with recombinant CD97 to FLS was studied by flow cytometry. Expression of CD46 and CD59, two other complement regulatory proteins, was also studied under the same conditions and dermal fibroblasts were investigated for comparison.

**Results:** Flow cytometric staining and quantitative real-time PCR demonstrated that CD55 is expressed at equal levels on cultured FLS of patients with RA, osteoarthritis, psoriatic arthritis and spondylarthritis. Functional relevant epitopes within the short consensus repeats 1 to 4 of CD55 were demonstrated to be accessible on FLS of the different arthritides. Stimulation of RA FLS with IL-1β and poly(I:C) induced a large and specific upregulation of CD55. This upregulation was not found in dermal fibroblasts, neither did it involve the related complement regulators CD46 and CD59. CD55 upregulation by poly(I:C) was demonstrated to be at least partly dependent on TLR3 activation and increased the binding capacity of FLS for CD97.

**Conclusions:** We identified TLR3 ligation as a potent inducer of CD55 upregulation on RA FLS. TLR3 is engaged by double-stranded RNA released from virus and necrotic cells, and has been demonstrated to stimulate pro-inflammatory gene expression in FLS (Brentano et al, Arthritis Rheum 2005). The upregulation of CD55 induced a higher binding capacity for CD97, and therefore could play a role in the retention of inflammatory cells into the synovium. We are currently investigating the expression of CD55 on RA FLS stimulated with soluble and cellular components of the synovial fluid.

**A105** DEVELOPMENT OF AN IN-VITRO MODEL OF RHEUMATOID CARTILAGE DESTRUCTION USING CROSS-LINKED TYPE II COLLAGEN MATRICES

1C Wunnau, ‘K Freyht, ‘N Pundd, ‘D Wendholt, ‘U Hansen, ‘B Dankbar, ‘T Pap. 1Division of Molecular Medicine of Musculoskeletal Tissue, Muenster, Germany; 2Division of Physiological Chemistry and Pathobiochemistry, Muenster, Germany

Synovial fibroblasts play an important role in rheumatoid joint destruction, and the interaction of these rheumatoid arthritis synovial fibroblasts (RASF) with components of the extracellular matrix (ECM) of cartilage is essential for their activation and aggressive behaviour. Therefore, in-vitro models of fibroblast invasion in RA need to model the specific properties of human articular cartilage. Here, we describe and characterise a modified matrix-associated transepithelial resistance invasion (MATRI) assay, in which freshly isolated type II collagen extracts cross-linked with type IX and XI collagen are used.

Human cartilage slices were homogenised on ice with a proteinase-containing extraction buffer and the suspension was cleared repeatedly. Electron microscopy was used to verify the intact cartilage-like superstructure of cross-linked type II collagen fragments in the supernatants before they were used for coating the inner filter cup compartment of a transwell system via specific cross-linkers. For the measurement of RASF invasion, we used the highly sensitive MATRI assay that is based on measuring the breakdown of electrical resistance created by a monolayer of Madin–Derby canine kidney (MDCK) C7 cells on the reverse side of the filter cup inserts. RASF obtained at joint replacement surgery were isolated by enzymatic digestion of synovial tissue and used in passages 4–8. For control purposes the invasion of RASF seeded on a stable mixture of collagen I and III was determined accordingly.

Transmission electron microscopy demonstrated the development of an intact type II collagen network following the extraction procedure. As measured by the MATRI assay with the cross-linked human type II collagen extract as an invasion barrier, RASF exhibited a clear invasiveness that showed comparable characteristics to those observed in the established mixture of collagen I and III. By contrast, no such invasion was seen with osteoarthritis synovial fibroblasts used as controls. Neither the human collagen extracts nor the cross-linker had any effect on the integrity of the MDCK-C7 monolayer.

We conclude that our novel MATRI assay that uses isolated type II collagen extracts cross-linked with type IX and XI collagen resembles the in-vivo cartilage composition in a far better way than conventional type I collagen-based assays, and serves as a suitable model for measuring precisely the invasive potential of RASF in vitro. This assay can therefore be used to analyse the interaction of fibroblasts with cartilage collagens and for screening anti-invasive compounds.

**A106** EXPRESSION OF SUMO-2/3 AND SENPS5 IN THE SYNOVIIUM OF RHEUMATOID ARTHRITIS PATIENTS

S Frank, S Strieholt, MA Peters, N Pundd, S Endor, T Pap. Division of Molecular Medicine of Musculoskeletal Tissue, University of Muenster, Muenster, Germany

SUMO-2/3 belongs to the small ubiquitin-related modifier family of proteins that have been implicated in many biological functions such as intracellular protein transport and the control of gene expression. Their function is regulated by SENPs, a nuleolar protein that preferentially deconjugates SUMO-2/3 from substrates but is also able to cleave SUMO-1 from target proteins. We were able to show previously that the overexpression of SUMO-1 in rheumatoid arthritis synovial fibroblasts (RASF) confers resistance against Fas-L-inducing apoptosis through a SUMO-1/SENP1-dependent mechanism. Based on similarities in the molecular
structures and function of SUMO-1 and the more recently described SUMO-2/3, we set out to analyse the expression of SUMO-2/3 and SENP5 in the synovial membranes and in synovial fibroblasts of rheumatoid arthritis (RA) patients compared with osteoarthritis.

Synovial tissue samples were obtained from RA and osteoarthritis patients at joint replacement surgery and were used for histological analysis as well as for the isolation of synovial fibroblasts. Using specific antibodies (Zymed, Abgent), the expression of SUMO-2/3 and SENP5 was analysed by immunohistochemistry in all synovial tissue specimens. The expression levels of SUMO-2/3 and SENP5 in RASF and osteoarthritic synovial fibroblasts (OASF) were compared by PCR and Western blot analysis. The subcellular localisation of SUMO-2/3 and SENP5 was investigated by immunocytochemical staining and confocal laser scanning microscopy.

Immunohistochemistry revealed a clear upregulation of SUMO-2/3 in all RA synovial tissue samples compared with osteoarthritis samples. SUMO-2/3 expression was most prominent in the superficial lining layer and in the sublining. RASF were identified as main SUMO-2/3 and SENP5-expressing cells by Western blot, in which RASF showed higher SUMO-2/3 and SENP5 protein levels than OASF. Immunocytochemical staining revealed that SUMO-2/3 is upregulated in RASF compared with OASF.

Our data indicate a disease-specific upregulation of SUMO-2/3 and SENP5 in the RA synovium. The data further suggest that like SUMO-1, SUMO-2/3 is expressed strongly in RASF. The simultaneous upregulation of SENP5, however, is in contrast to the SENP1 data and suggests a distinct function of SENP5 in RASF. Based on the established role of SUMO in mediating the resistance of RASF against apoptosis, we hypothesise that SUMO-2/3 is a novel player contributing to specific features of activation in RASF and, thus, to the disease process of RA.

In vitro, early cultured RASF revealed a low proliferation rate. The proliferation rate decreased further over time, particularly when RASF became confluent. RASF expressed Fas, TRAIL-R1 and TRAIL-R2, whereas TRAIL-R3 and TRAIL-R4 were not detectable. However, the susceptibility to Fas ligand and TRAIL-induced apoptosis was influenced significantly by the proliferation rate of RASF. In particular, RASF with a decreased proliferation rate were more sensitive to Fas ligand and TRAIL-induced apoptosis than higher proliferating RASF. Moreover, RASF that were synchronised with hydroxyurea in the G0/G1 phase were more sensitive to TRAIL-induced apoptosis than synchronised RASF in the S phase or in the G2/M phase. Furthermore, proliferating RASF arrested in the S phase or in the G2/M phase with ITS-supplemented medium were less sensitive to Fas ligand and TRAIL-induced apoptosis than RASF in the G0/G1 phase.

Our data confirm that the susceptibility of RASF to Fas ligand and TRAIL-induced apoptosis depends on the cell cycle. These results may explain the conflicting data on the ability of RASF to undergo Fas ligand and TRAIL-mediated cell death.

Background and Objectives: One of the suggested mechanisms of microplacental thrombosis in patients with antiphospholipid syndrome is a phospholipid antibody-mediated disruption of the annexin A5 anticoagulant shield. Annexin has been shown spontaneously to form trimers that self-organise into two-dimen- sional crystals, with p3 or p6 symmetry, after binding on membrane. The potent anticoagulant properties of annexin are suggested to be a consequence of this crystallisation, which forms a lattice of annexin over the phospholipid surface, blocking its availability for coagulation reactions. The aim of our study was to find out if such crystallisation occurs on supported phospholipid bilayers (SPB) prepared from naturally derived phospholipids.

Materials and Methods: Atomic force microscopy (AFM) was used for the observation of crystallisation, for its unique ability to image biological macromolecules in their native aqueous environment, with a nanometer resolution. Different SPB were prepared of freshly sonicated vesicles, formed under low pressure in the presence of nitrogen. Naturally derived, non-homogeneous phospholipids and synthetic phospholipids were used separately using phosphatidylserine (50%) and phosphatidylcholine in heps buffer saline.

Results: For the first time, crystallisation of annexin A5 on SPB, consisting of naturally derived, non-homogenic phospholipids, has been observed and documented by images and force–distance curves. We have proved by AFM analyses, that annexin A5 is able to self-assemble on SPB obtained from natural inhomogeneous phospholipids in two different crystallisation forms, termed p3 and p6. Moreover, we have observed that the crystallised annexin A5 layer on SPB could be elastically deformed, but could not be ruptured, as is the case with bare SPB.

Conclusions: A demonstration that native annexin A5 is able spontaneously to crystallise on naturally derived, inhomogeneous phospholipids supports the putative role of annexin A5 crystal structures as a possible antithrombotic shield. With observed crystallisation of annexin A5 on naturally derived phospholipids we came closer to the physiological conditions in the human body and to understanding the molecular basis of vascular and microplacental thrombosis.
8. Cartilage and bone destruction

A109 ABSENCE OF ALPHA2 INTEGRIN ALTERS MATRIX METALLOPROTEINASE EXPRESSION AND TUMOUR NECROSIS FACTOR-DEPENDENT INFLAMMATORY CARTILAGE DESTRUCTION

†MA Peters, †S Strieholt, †S Frank, †D Wendholt, †A Korb, †G Kollas, †B Eckes, †T Pap. Division of Molecular Medicine of Musculoskeletal Tissue, University of Muenster, Muenster, Germany; 2Department of Internal Medicine D, University of Muenster, Muenster, Germany; 3Institute of Immunology, Biomedical Sciences Research Center, Vari, Greece; 4Department of Dermatology, University of Cologne, Cologne, Germany

Integrins are the main receptors for cell–matrix interactions and integrin signalling is critical for a variety of cellular functions such as adhesion, cell spreading, chemotaxis and inflammatory responses. α2 Integrin functions as the major receptor for type I collagen on a number of different cells, among them both fibroblasts and inflammatory cells. Although α2 integrin-deficient mice show no obvious abnormalities apart from partial defects in platelet interaction, it was shown that α2 integrin contributes to the induction of matrix metalloproteinases (MMP) in tissue remodelling. Based on the hypothesis that under stress conditions such as chronic inflammation, α2 integrin may be involved in the activation of synovial cells, we investigated the role of α2 integrin in inflammatory arthritis.

To determine the role of α2 integrin in tumour necrosis factor (TNF)-mediated joint disease, we crossed α2-deficient mice with arthritic human TNFα transgenic (hTNFtg) mice. Clinical signs of arthritis and weight as well as the histological degree of synovitis and cartilage destruction in hind paws were investigated using standard clinical evaluation and histomorphometric analysis. In addition, we analysed serum levels of cytokines and MMP and used an established in-vitro assay to investigate the role of the α2 subunit in the attachment of synovial fibroblasts from these mice to healthy and IL-1-damaged articular cartilage. Synovial fibroblasts from all genotypes were also analysed for MMP production.

Overall, the knockout of α2 integrin in hTNFtg mice resulted in slightly improved clinical signs and symptoms compared with hTNFtg mice. We found that hTNFtg/α2(−/−) mice had less paw swelling, increased grip strength and a less pronounced weight loss. Histological analysis revealed that hTNFtg/α2(−/−) mice had a decrease in synovial inflammation compared with hTNFtg mice. Interestingly, we found an increase in MMP3 and MMP9 serum levels in α2-deficient mice. Analysing synovial fibroblasts from α2-deficient mice, we also found an upregulation of MMP3 expression compared with wild-type synovial fibroblasts. This upregulation was similar but somewhat less pronounced when synovial fibroblasts from hTNFtg/α2(−/−) mice were compared with fibroblasts from hTNFtg animals. In addition, we found an altered attachment of synovial fibroblasts lacking α2 integrin to IL-1-treated cartilage pieces in vitro.

Our findings suggest that although α2 integrin appears to be dispensable for normal development, the loss of α2 integrin leads to a decrease in inflammation and bone destruction in an animal model of inflammatory arthritis.

A110 ANTI-DKK1 TREATMENT PROTECTS TUMOUR NECROSIS FACTOR-TRANSGENIC MICE FROM INFLAMMATORY OSTEOPOROSIS

G Ruiz, K Polzer, W Baum, G Krönke, R Axmann, G Schett, J Zwerina. Department of Internal Medicine 3, University of Erlangen-Nuremberg, Erlangen, Germany

Objectives: Chronic inflammation is a major risk factor for systemic bone loss leading to osteoporotic fracture and substantial morbidity and mortality. Inflammatory cytokines are thought to play a key role in the pathogenesis of inflammation. Recently, the Wnt signalling pathway was shown to regulate joint destruction in animal models of arthritis. Therefore, we analysed whether stimulation of the Wnt pathway with an anti-DKK1 antibody alters inflammatory bone loss in an animal model of arthritis.

Methods: Six-week-old human tumour necrosis factor transgenic (hTNFtg) mice were treated with the following agents for 4 weeks: vehicle (phosphate-buffered saline); an anti-tumour necrosis factor (TNF) antibody (10 mg/kg three times a week); a rat antibody to mouse DKK-1 (10 or 30 mg/kg three times a week) or a combination of anti-TNF and anti-DKK-1. Systemic bone mineral density was analysed by micro computed tomography and bone histomorphometry. Calcein labelling was used for dynamic histomorphometry. In vitro, isolated osteoblasts were stimulated with TNF and analysed functionally.

Results: Untreated hTNFtg mice developed severe bone loss associated with disturbed bone microarchitecture. Bone trabeculae were both thinner and decreased in numbers resulting in an increased trabecular separation. Bone histomorphometry revealed an increase in bone resorption and decreased bone formation responsible for bone loss in hTNFtg mice. Treatment with anti-TNF partly reversed systemic bone loss. Despite leaving synovial inflammation untouched, anti-DKK1 treatment dose-dependently inhibited TNF-induced bone loss. The combination of anti-TNF and anti-DKK1 was the most efficient treatment strategy. In contrast to anti-TNF, anti-DKK1 treatment led not only to a decrease in osteoclast formation but also an increase in bone formation. In-vitro osteoblast cultures confirmed these findings.

Conclusion: These data indicate that modulation of the Wnt pathway essentially influences TNF-mediated bone loss. Despite TNF-mediated inflammatory arthritis, systemic bone is protected by treatment with an anti-DKK1 antibody.

A111 ARTICULAR CARTILAGE MINERALISATION IN HUMAN OSTEOARTHRITIS

†M Fuerst, †J Bertrand, †L Lammers, †W Rüther, †T Pap. Department of Orthopedic Surgery, University Medical Center Hamburg, Eppendorf, Germany; 2Institute of Experimental Musculoskeletal Medicine, University Hospital Münster, Münster, Germany; 3Institute of Mineralogy, University Hospital Münster, Münster, Germany

Background: In enchondral ossification, hypertrophic chondrocytes are specialised to mineralise the extracellular matrix. Chondrocyte hypertrophy and mineralisation occur in osteoarthritis, but the question of whether chondrocyte hypertrophy is linked to crystal formation and to the pathogenesis of osteoarthritis remains incompletely understood. Here, we report on the prevalence and composition of calcium crystals in end-stage osteoarthritis and how they relate to disease severity.

Methods: 120 patients with end-stage osteoarthritis consecutively undergoing total knee replacement were prospectively evaluated. As controls, five patients with bone tumour and no affection of the knee joint were included. Cartilage calcification was analysed by digital contact radiography (DCR) and electron microscopy (FE-SEM), and its relationship to histological changes and chondrocyte maturation in cell culture was determined. The expression of different genes involved in matrix calcification, such as ENPP1, ANK and TNAF, was investigated.

Findings: DCR revealed mineralisation in all 120 cartilage specimens. The amount of cartilage mineralisation showed a significant inverse correlation with the clinical van der Heijde modified Sharp knee score used for preoperative evaluation. FE-SEM analysis showed that, in all probes, basic calcium phosphates (BCP) such as apatite were the prominent minerals. The capability of chondrocytes to mineralise in vitro correlates positively with the amount of mineralisation in articular cartilage. We were able to show a positive correlation between increasing mineralisation and collagen X expression. The induction of hypertrophy in healthy human articular cartilage resulted in chondrocyte matrix mineralisation.
Interpretation: The data indicate that articular cartilage mineralisation by BCP formation is an indissociable process in the development of osteoarthritis, and this impacts the clinical consequences and therapeutic strategies for osteoarthritis. As suggested by the nature of the crystals, the correlation with hypertrophy markers and in-vitro calcification, calcification might be linked to a mechanism of enchondral ossification.

Background and Objectives: Strategies for treating rheumatoid arthritis (RA) patients aim both to suppress inflammation and to prevent future joint destruction. As current predictors of joint destruction have low specificity and ongoing inflammation is common despite low C-reactive protein (CRP) and disease activity measured by magnetic resonance imaging (MRI), and to assess whether the biomarker levels at baseline were predictive of progression in erosions measured by MRI and conventional radiographs (CR).

Methods: A cohort of 84 early RA patients (disease duration <1 year, mean age 58.4 years, 73.9% women, 55% citric citrullinated peptide positive) was followed longitudinally with assessment at baseline, 3, 6 and 12 months including clinical measures of disease activity and inflammation measured by magnetic resonance imaging (MRI), and to assess whether the biomarker levels at baseline were predictive of progression in erosions measured by MRI and conventional radiographs (CR).

Results: Levels of sYKL-40, sCRP and sMMP3 were longitudinally associated (p<0.05) with disease activity (DA528) and MRI measures of inflammation (RAMRIS erosions range 0–150, synovitis range 0–9 and bone marrow oedema range 0–45). The CR were scored according to the van der Heijde modified Sharp score. With the exception of C-reactive protein (Nefelometric; Dade Behring, Newark, DE, USA), all measurements were by ELISA: serum osteoprotegerin and sRANKL (Biomedica Medizinprodukte, Vienna, Austria), serum C-terminal crosslinked telopeptide (CTX) type I and uCTX II (Nordic Bioscience, Herlev Denmark), sYKL-40 (Quidel, San Diego, CA, USA), serum cartilage oligomeric matrix protein (Anamari Medical AB, Lund, Sweden) and serum matrix metalloproteinase (MMP) 3 (Bender MedSystems, Vienna, Austria). Multiple linear mixed model analyses for repeated measures were performed to examine longitudinal associations between the biomarkers and disease activity and inflammation and multiple linear (MRI) and logistic (CR) regression analyses and to examine predictors of progression of damage.

Conclusion: Serial measurements of MMP3 and YKL-40 were consistently associated with disease activity and MRI scores of inflammation independent of CRP during the study. The bone marker sCTX I was a predictor of erosive progression measured by MRI and CR. The cartilage marker uCTX II predicted progression measured by CR. This study indicates that biomarkers might contribute to monitoring disease activity and joint destruction in the follow-up of RA patients.

Background and Objectives: Bortezomib is a proteosome inhibitor, which is known as a clinically potent antimyeloma agent. Bortezomib has been shown to inhibit osteoclastogenesis directly in humans as well as to increase osteoblasts in bortezomib-treated patients, which indicates that bortezomib has bone-anabolic activity. We wanted to analyse the influence and therapeutic effect of bortezomib in an inflammatory mouse model of arthritis.

Materials and Methods: Heterozygous human tumour necrosis factor alpha transgenic mice (hTNFtg mice, strain tg197) were treated with 20 mg/kg bortezomib or phosphate-buffered saline, which served as a control, three times per week intravenously. Mice were assessed weekly for clinical signs of arthritis. Hind paws were analysed by standardised staining for synovial inflammation, bone erosions and cartilage damage. Osteoclasts from hTNFtg mice were obtained from bone marrow and stimulated with different concentrations of bortezomib. Furthermore, stimulated osteoclasts were analysed by Western blot and real-time PCR in more detail.

Results: Bortezomib-treated hTNFtg mice showed in-vivo accelerated areas of inflammation and bone erosions in the hind paws (hTNFtg 0.13 ± 0.02 mm²; hTNFtg + bortezomib 0.42 ± 0.09 mm²) correlating with a significant increase in the number of osteoclasts (hTNFtg 55 ± 5; hTNFtg + bortezomib 94 ± 19). There was no statistical difference in cartilage damage between the treated and untreated groups of mice. Ex vivo analysis of bortezomib-treated osteoclasts shows an increase in the differentiation of monocytes into osteoclasts when they were stimulated with concentrations of 10 ng/ml or lower, whereas concentrations higher than 1 μg/ml resulted in complete apoptosis of monocytes. The activity of osteoclasts was visualised using a resorption assay. Bortezomib-stimulated osteoclasts caused resorption pits of greater extent than osteoclasts without stimulation. Bortezomib-treated osteoclasts revealed greater expression of TRAF6, c-fos and Nfat-c messenger RNA, which could be verified by Western blots of these proteins.

Conclusion: Bortezomib increases osteoclastogenesis and bone destruction in an inflammatory mouse model of arthritis.

Background and Objectives: Different inflammatory joint diseases have distinct patterns of bone damage, with pronounced erosions in rheumatoid arthritis (RA), a combination of bone destruction and formation in psoriatic arthritis (PsA) and dominant new bone formation in spondyloarthritis (SpA). Although the underlying molecular mechanisms remain poorly understood,
blocking of Dickkopf-1 (DKK-1), an inhibitor of the Wnt pathway, reverses the bone-destructive pattern to a bone-forming pattern in experimental arthritis. Therefore, we analysed DKK-1 expression in the inflamed peripheral joints of different types of inflammatory arthritis ex vivo and in fibroblast-like synoviocyte (FLS) cultures in vitro.

Materials and Methods: Synovial fluid (SF) was obtained from actively inflamed knee joints of RA (n = 45), non-psoriatic SpA (n = 38) and PsA (n = 35) patients. FLS cultures from RA (n = 5), SpA (n = 6) and PsA (n = 5) patients were starved for 24 h in medium with 1% fetal calf serum followed by stimulation with tumour necrosis factor alpha (TNFα; 10 ng/ml), IL-1β (10 ng/ml) and IL-6 (50 ng/ml)/IL-6R (50 ng/ml) for 72 h. The concentrations of IL-6, DKK-1, TNFα and IL-1β were determined by ELISA.

Results: Compared with the previously reported serum levels of 3.1 ± 1.9 pg/ml in RA and 5.8 ± 0.3 pg/ml in axial SpA, the SF levels of DKK-1 were 263.6 ± 119.5 pg/ml in RA, 276.4 ± 93.5 pg/ml in SpA and 411.9 ± 163.1 pg/ml in PsA. There was no statistical difference between the disease groups but there was a striking variability within each cohort. As TNFα stimulates DKK-1 production, we also assessed SF pro-inflammatory cytokine levels. Despite the similar DKK-1 levels, both TNFα and IL-1β levels but not IL-6 levels were significantly higher in RA than in SpA. Accordingly, TNFα and IL-1β levels were not correlated with DKK-1 levels. In contrast, there was a striking inverse correlation between DKK-1 and IL-1β in both RA (r = -0.37, p = 0.013) and SpA (r = -0.35, p = 0.034). In agreement with the SF ex-vivo studies, the DKK-1 level in FLS from RA patients was strongly elevated by TNFα (but not IL-1β) and suppressed by IL-6. A similar trend was observed in FLS from SpA and PsA patients, although the DKK-1 basal expression level in these groups was higher than in RA. These data will be confirmed immunohistochemically on synovial tissue biopsies.

Conclusion: DKK-1 is abundantly expressed in the inflamed joints of both destructive and remodelling forms of arthritis. The differential regulation of DKK-1 by TNFα and IL-6 is under further investigation as it may determine the pattern of inflammation-induced tissue remodelling in arthritic joints.

A115 GDF5 HAPLOINSUFFICIENCY INCREASES SUSCEPTIBILITY TO OSTEOARTHRITIS IN MICE

M Doans, R Lories, F Luyten. Laboratory for Skeletal Development and Joint Disorders, Katholieke Universiteit Leuven, Leuven, Belgium

Background and Objectives: Growth and differentiation factor 5 (GDF5) is a member of the transforming growth factor beta and bone morphogenetic protein superfamily and plays a role in limb development and joint formation. Postnatally, GDF5 is expressed in different joint structures and appears to be important for the healing of bones, tendons, ligaments and meniscus after injury. A polymorphism in the human GDF5 gene is associated with susceptibility to osteoarthritis. The aim of this study was to investigate the effect of GDF5 hypomorphism on the development of joint disease in mice to understand further the underlying mechanisms of its association with osteoarthritis.

Material and Methods: We used heterozygous brachypodism mice (Gdf5Bp/+), in which a spontaneous mutation resulted in the functional deletion of one allele and control littermates in different osteoarthritis models. Chemically, osteoarthritis was induced by an intra-articular injection of collagenase VII (2 U/μl) (collagenase) or papain 1%/l-cystein 0.08 mol (phosphate-buffered saline in contralateral knee) (papain). Surgically, osteoarthritis was induced by destabilisation of the medial meniscus (DMM). Spontaneously occurring osteoarthritis was accelerated by forced treadmill running for 5 weeks (treadmill). Mice were killed after 21 days (collagenase), 7 days (papain), 8 weeks (DMM) and 5 weeks (treadmill). The severity of disease was determined by histological scores (0 to 3) on haematoxylin and eosin stained sections throughout the knee (five sections at 100 μm distance) for synovial hyperplasia, infiltration and fibrosis. Cartilage damage was measured by digital image analysis using safranin O-stained sections (five sections at 100 μm distance) quantifying proteoglycan content (collagenase, papain and treadmill) or was scored using the Osteoarthritis Research Society International (OARSI) score (DMM).

Results: Gdf5Bp/+ mice had an increased susceptibility to develop osteoarthritis in instability-induced osteoarthritis models (collagenase and DMM) and in the treadmill model. Collagenase injection induced severe joint instability, characterised by cartilage degradation, osteophyte formation and mild synovitis in wild-type animals; in heterozygous mice, collagenase treatment induced comparable defects, but moreover the contralateral knee was also affected. In the mild instability model of DMM, Gdf5Bp/+ mice developed a more severe osteoarthritis characterised by an increased formation of osteophytes and loss of cartilage in the destabilised knee. In the treadmill model, the incidence of spontaneously occurring osteoarthritis was higher in the Gdf5Bp/+ group and osteoarthritis was characterised by osteophytes and loss of cartilage, mainly at the medial tibia. In an enzymatic injury model of osteoarthritis (papain), however, GDF5 deficiency did not alter severity, or cartilage breakdown.

Conclusions: These results show that Gdf5Bp/+ mice are more prone to osteoarthritis, which is in line with human genetic findings. Osteoarthritis in mice appears to be associated with increased joint instability.

A116 IN-VITRO SPONTANEOUS OSTEOCLASTOGENESIS IS NOT CRUCIALLY DEPENDENT ON T LYMPHOCYTES

1D Baeten, 1B Vandooren, 1L Melis, 1E Vees, 1P Tak. 1Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; 2Ghent University Hospital, Ghent, Belgium

Objective: In-vitro spontaneous osteoclastogenesis from peripheral blood mononuclear cells (PBMC) is increased in diseases with excessive bone loss. Here, we reassessed the role of T lymphocytes in this process.

Methods: Fresh or cryopreserved PBMC obtained from healthy individuals and patients with rheumatoid arthritis (RA), psoriatic arthritis (PsA) and non-psoriatic spondyloarthropathy (SpA) were cultured at high density and stained for tartrate-resistant acid phosphate (TRAP). Resorption of mineralised matrix was assessed by a dentine disc assay. CD14-positive monocytes and CD3-positive T cells were selected using magnetically labelled antibodies.

Results: Numerous multinuclear TRAP-positive, dentine-resorbing osteoclasts developed spontaneously from fresh PBMC of healthy individuals. This process was abrogated by T-cell depletion and restored by exogenous macrophage colony-stimulating factor (M-CSF) and RANKL, indicating the important role of T cells in spontaneous osteoclastogenesis in vitro. Using physiological freezing and thawing as a model to activate PBMC, spontaneous osteoclastogenesis was significantly increased in cryopreserved versus fresh cells. In these conditions, spontaneous osteoclastogenesis was not dependent on T lymphocytes as it was not influenced by T-cell depletion and persisted in purified CD14-positive cell cultures supplemented with M-CSF and RANKL. In contrast to studies with fresh PBMC, spontaneous osteoclastogenesis in these conditions did not appear to be clearly different between healthy subjects and arthritic patients.

Conclusion: Spontaneous osteoclastogenesis in vitro is dependent on either T lymphocytes or the direct activation of monocytes cells according to test conditions. This variability warrants better validation of the relevance of this functional test for in-vivo osteoclastogenesis.
**A117** INDUCTION OF OSTEOCLAST-ASSOCIATED RECEPTOR, A KEY OSTEOCLAST CO-STIMULATION MOLECULE, IN RHEUMATOID ARTHRITIS

1 S Herman, 1 R Mueller, 1 G Kronke, 1 J Zwerina, 1 K Redlich, 1 U Mueller-Ladner, 2 G Schett. 1 Department of Internal Medicine 3, University of Erlangen-Nuremberg, Erlangen, Germany; 2 Institute of Medical Physics, University of Erlangen, Germany; 3 Institute of Medical III, Medical University of Vienna, Vienna, Austria; 4 Justus-Liebig-University, Bad Nauheim, Germany

**Objectives:** To define the role of co-stimulation in osteoclast differentiation during inflammatory arthritis. Osteoclast-associated receptor (OSCAR) is a new osteoclast-specific receptor and is of key importance in the co-stimulation process of osteoclasts.

**Methods:** OSCAR expression was assessed in peripheral blood monocytes and in the serum of patients with rheumatoid arthritis (RA) and was related to disease activity. Further expression patterns of OSCAR were analysed before and after treatment with anti-tumour necrosis factor alpha (TNFα) antibodies. In-vitro osteoclast experiments revealed the role of OSCAR in human osteoclastogenesis with special regard to the relevance of OSCAR under pathological autoimmune circumstances.

**Results:** Flow cytometry analysis revealed an enhanced expression of OSCAR on peripheral blood monocytes of patients with RA. Soluble OSCAR in serum could be detected by ELISA and revealed a lower concentration in patients compared with healthy controls. Both surface and soluble OSCAR was correlated with disease activity and acute phase reactants. Moreover, monocytes with a high OSCAR surface expression revealed an enhanced potential to differentiate into osteoclasts. Finally, TNFα was identified as the main inducer of OSCAR expression in monocytes.

**Conclusion:** These data suggest that the osteoclast co-stimulation pathway is activated in RA. OSCAR is induced in monocytes of RA patients, facilitating their differentiation into osteoclasts and bone resorption.

**A118** P38ALPHA IS A KEY REGULATOR OF ARTHRITIS-INDUCED BONE EROSION

1 C Böhm, 2 A Kilian, 1 MM Zais, 3 S Finger, 1 A Hess, 4 K Engelke, 5 EF Wagner, 2 G Kollars, 2 G Krötka, 1 J Zwerina, 1 G Schett, 1 JP David. 1 Department of Internal Medicine 3, Rheumatology and Immunology, University of Erlangen-Nuremberg, Erlangen, Germany; 2 Institute of Experimental and Clinical Pharmacology and Toxicology, University of Erlangen-Nuremberg, Erlangen, Germany; 3 Division of Endocrinology, Diabetes, and Bone Diseases, Medicine III, Technical University Dresden, Dresden, Germany; 4 Institute of Medical Physics, University of Erlangen-Nuremberg, Erlangen, Germany; 5 Spanish National Cancer Research Center, CNIO, Madrid, Spain; 6 Institute of Immunology, Alexander Fleming Biomedical Sciences Research Center, Vari, Greece

**Background and Objectives:** Pharmacological inhibitors have provided evidence for the key role of p38 mitogen-activated protein (MAP) kinase in osteoclast differentiation and in inflammation-induced bone loss. However, these inhibitors block more than one of the four p38 isoforms, usually alpha and beta, and sometimes also other kinases such as JNK3. Here we address the role of p38α in inflammation-induced arthritis.

**Materials and Methods:** The expression of p38 in osteoclast lineage is analysed by Western blotting and quantitative PCR. The role of p38α in inflammation-induced arthritis is analysed following the Cre-mediated deletion of p38α in the haematopoietic cells of the human transgenic tumour necrosis factor alpha (TNFα) transgenic mouse, a mouse model for arthritis. Clinical parameters and histology of the paw are compared. Bone parameters are measured by both micro computer tomography and bone histology. Finally, the effect of p38 deletion or small interfering RNA-mediated knock down of p38α on the differentiation of monocytes into bone-resorbing cell osteoclasts is analysed in vitro.

**Results:** We now show that p38α is the main p38 isoenzyme expressed in osteoclast precursors and in mature cells. p38α as well as its downstream substrates were phosphorylated in osteoclast progenitors stimulated by TNFα. Using Cre-mediated conditional gene inactivation we demonstrated that mice lacking p38α in the haematopoietic cells were protected against TNFα-induced bone destruction at the site of inflammation as well as against TNFα-mediated systemic bone loss. The protection was caused by decreased osteoclast numbers in vivo. The phenotype was cell autonomous because, similar to p38α-deficient cells, knock-down of p38α in monocytes resulted in a decreased osteoclast differentiation in vitro.

**Conclusions:** Our data have shown that developing specific inhibitors of the alpha isoenzyme of p38 would be beneficial for the treatment of inflammation-induced bone destruction as observed in rheumatoid arthritis.

**A119** SCAVENGER RECEPTOR CLASS AI/II DETERMINES SEVERE CARTILAGE DESTRUCTION (METALLOPROTEINASE-MEDIATED MATRIX BREAKDOWN AND CHONDROCYTE DEATH) DURING ANTIGEN-INDUCED ARTHRITIS

1 P van Lent, 1 W Holkens, 2 A Blom, 1 S Grevers, 3 A Sletjøs, 4 M De Winther, W van den Berg. 1 Experimental Rheumatology and Advanced Therapeutics, Radboud University Medical Centre, Nijmegen, The Netherlands; 2 Molecular Genetics, Cardiovascular Research Institute Maastricht, Maastricht, The Netherlands

**Background and Objectives:** Scavenger class A receptors (SR-A) are particularly expressed by macrophages and bind and internalise a broad range of molecules and particles carrying pathogen-like molecular patterns, such as bacterial endotoxins, apoptotic bodies, proteoglycans and oxidised low-density lipoprotein. The objective was to investigate the role of SR-A I/II in mediating severe cartilage destruction during antigen-induced arthritis (AIA).

**Materials and Methods:** AIA was induced in knee joints of SR-A I/II−/− mice and their wild-type controls. Joint inflammation and cartilage destruction were measured using histology of total knee joints. Matrix metalloproteinase (MMP)-mediated neoepitopes were measured with immunohistochemical staining using anti-MMP antibodies. Chondrocyte activation was measured by immunohistology and antibodies against the activation marker S100A8. Chondrocyte death was determined by measuring empty lacunae in cartilage of haematoxylin and eosin-stained sections, respectively. Messenger RNA levels were determined in inflamed synovium using quantitative reverse transcriptase PCR.

**Results:** mRNA levels of SR-A I/II were elevated in inflamed synovium during antigen-induced arthritis (days 1, 3, 5 and 7, respectively, 5, 9, 11 and fivefold). SR-A I/II proteins were clearly expressed on macrophages at days 7 and 14 after AIA. Development of immunity (T-cell responses and antibody titres) against methylated bovine serum albumin was comparable between wild-type and SR-A I/II−/− mice. At days 2, 8 and 14 after AIA induction, joint inflammation (exudates/infiltrate) was not different between the two strains. In wild-type mice at day 14 after AIA induction, severe cartilage destruction was found in multiple cartilage surfaces (lateral and medial tibia and femur) of the inflamed knee joint. MMP-mediated matrix destruction ranged between 40% and 60% of cartilage surfaces, chondrocyte activation occurred in 40–70% of cartilage surfaces and chondrocyte death was prominent in 40–75% of the cartilage surfaces. In striking contrast, in SR-A I/II−/− mice, although joint inflammation was comparable, all parameters of severe cartilage destruction were almost completely absent. mRNA levels of MMP-13 and MMP-14 but not MMP-2, 3 and 9 were significantly lower at day 7 in inflamed synovia of SR-A−/− mice.

**Conclusion:** SR-A I/II are crucial receptors involved in mediating severe cartilage destruction during antigen-induced arthritis probably by activating MMP.
SYNDECAN-4 REGULATES MATRIX METALLOPROTEINASE TYPE 3-DEPENDENT ACTIVATION OF ADAMTS-5 IN OSTEOARTHRITIC CARTILAGE

1J Bertrand, 4E Echtemeyer, 4R Dreier, 1K Neugebauer, 2T Pap. Division of Molecular Medicine of Musculoskeletal Tissue, University Hospital of Muenster, Muenster, Germany; 2Department of Anaesthesiology, Medical School Hanover, Hanover, Germany; 3Department of Physics, Chemistry and Pathobiocchemistry, University Hospital of Muenster, Muenster, Germany

The cleavage of aggrecan is an important step in the breakdown of cartilage matrix in osteoarthritis. ADAMTS-5 has been shown to be involved in the loss of proteoglycans, but the mechanisms of ADAMTS-5 activation during cartilage remodelling are poorly understood. Here, we analysed the role of syndecan-4 in regulating the expression and activation of ADAMTS-5 in human osteoarthritic cartilage and in a murine model.

Cartilage specimens were obtained from 120 patients with knee osteoarthritis at joint replacement surgery and analysed for syndecan-4 and collagen X expression. For functional analysis, osteoarthritic-like changes were induced in syndecan-4 knockout mice (n = 9) and wild-type animals (n = 8) by surgically achieved joint instability. To verify the data of the syndecan-4 knockout mice and exclude developmental effects, wild-type mice (n = 8) were treated with intra-articular injections of a blocking antibody against syndecan-4 following the induction of osteoarthritis. The histological severity of osteoarthritis as well as the MMP-3 expression was assessed in histological sections of all animals. ADAMTS-5 activity was determined by a sensitive aggrecanase assay. The effect of the MMP-3 inhibitor NGH on aggrecan cleavage was analysed in murine cartilage explants. Changes in IL-1-induced expression of MMP, ADAMTS and TIMP were measured by reverse transcriptase PCR. Syndecan-4-dependent changes in ERK phosphorylation after IL-1 treatment were determined by Western blot.

During the development of murine osteoarthritis, syndecan-4 was induced specifically in type X collagen-expressing chondrocytes. The loss of syndecan-4 in the knockout mice or treatment of wild-type animals with syndecan-4 antibodies nearly completely protected from a loss of proteoglycans. This correlated with a reduced MMP-3 expression in the cartilage of syndecan-4 knockout mice and in mice treated with anti-syndecan-4 antibodies. Proteoglycan loss was induced by IL-1 in cartilage caps isolated from 3–8-week-old wild-type and syndecan-4 knockout mice, and ADAMTS-5 expression was found to be upregulated by IL-1 equally in both groups. However, proteoglycan loss was reduced significantly in syndecan-4 knockout cartilage or cartilage treated with anti-syndecan-4 antibodies. In the supernatant of syndecan-4 knockout cartilage, we found a reduced presence of activated ADAMTS. Cartilage that was treated with NGH showed a reduced ADAMTS-5-mediated proteoglycan cleavage. We found a reduction of phosphorylated ERK in syndecan-4 knockout cartilage after IL-1 treatment.

Our data demonstrate that syndecan-4 is functionally involved in cartilage degradation by hypertrophic osteoarthritic chondrocytes through inhibiting the activation of ADAMTS-5-mediated aggrecan cleavage. ADAMTS-5 activation depends on both direct interaction with syndecan-4 on the surface of osteoarthritic chondrocytes and syndecan-4-regulated synthesis of MMP-3, which is ERK dependent. Inhibition of syndecan-4 may, therefore, constitute a promising strategy to interfere with osteoarthritic cartilage damage.

THE ROLE OF THE MULTIFUNCTIONAL ADAPTER PROTEIN P62/SOSTM1 IN INFLAMMATORY ARTHRITIS

1A Korb, 1A Hillmann, 3S Burgis, 4G Kollia, 2T Pap, 4T Weide, 1A Gissner, 1H Pavenski, 1Department of Nephropathy and Rheumatology, University Hospital Muenster, Muenster, Germany; 2Division of Molecular Medicine of Musculoskeletal Tissue, University Hospital Muenster, Muenster, Germany; 3Department of Immunology, University Erlangen, Erlangen, Germany; 4BSRC Fleming, Van, Greece

Activation of the RANK–nuclear factor kappa B (NF-κB) pathway is important for osteoclastogenesis, and pro-inflammatory cytokines such as IL-1 and tumour necrosis factor alpha (TNFα) enhance osteoclastogenesis in rheumatoid arthritis (RA) at multiple levels. The ubiquitin-binding, multi-adaptor protein p62/SOSTM1 is a prominent example for adaptor proteins that modulate cytokine function either through direct interaction with downstream targets or through altering protein ubiquitination and degradation. It contains a ubiquitin-binding domain that has been implicated in multiple functions of p62 and can modulate cytokine pathways such as RANKL-induced IKK activation through complex formation with TRAF6 and aPKC. However, the question of whether p62 regulates osteoclastogenesis during chronic inflammation and whether direct protein interaction through TRAF6 and aPKC or ubiquitin-binding mediates these effects is unknown.

Therefore, mice were generated that carry a mutant p62 with a functional ubiquitin-binding domain but defective signal transduction domains (p62aaD69–251). Cells from these mice were analysed for p62 function, and the mice were interbred with arthritic hTNFtg mice, a mouse model for RA. All mice (wild-type, hTNFtg, p62aaD69–251 and hTNFtg/p62aaD69–251/wt) were scored for paw swelling, grip strength and weight once weekly for 14 weeks. Joints of 14-week-old mice of all genotypes were embedded into paraffin, stained with toluidine blue and analysed by histomorphometry for the extent of joint inflammation, proteoglycan loss and erosions in cartilage and bone. The expression of p62 was studied by immunohistochemistry in these sections and the number of osteoclasts was assessed by TRAP staining. Osteoclastogenesis was studied in vitro using an established osteoclast formation assay.

By immunohistochemistry, increased expression of p62 was found in the inflamed synovial tissues of hTNFtg mice. Analysis of p62aaD69–251 mice showed a significantly increased osteoclastogenesis in vitro, but only minor changes in vivo, suggesting that under physiological conditions, regulatory mechanisms can compensate for the lack of the signal transduction domains of p62. When challenged in the hTNFtg background, however, the lack of these p62 domains had significant effects. The majority of hTNFtg/ p62aaD69–251/aaD69–251 mice was embryonically lethal. hTNFtg mice that were heterozygous for this p62 mutation (hTNFtg/ p62aaD69–251/wt) showed normal weight and fertility but a dramatic increase in the severity of joint damage as determined both clinically and by histomorphometry. This was accompanied by an increase in the number and size of osteoclasts in vivo.

In summary, our data suggest that p62 is an important regulator of TNFα-mediated joint damage. They indicate that the loss of the TRAF6 and aPKC-binding domains of p62 is dispensable for normal development but has important consequences for osteoclastogenesis under inflammatory conditions.

9. Animal models

A LONGITUDINAL STUDY OF PATHOLOGICAL CHANGES IN JOINTS OF RATS WITH PRISTANE-INDUCED ARTHRITIS

1MH Hoffmann, 1S Herman, 2G Zanoni, 2R Hopf, 1B Niederreiter, 1G Steiner. Division of Rheumatology, Medical University of Vienna, Vienna, Austria; 2AUVA Research Center for Traumatology, Vienna, Austria

Background and Objectives: Pristane-induced arthritis (PIA) in the rat has been described as an animal arthritis model closely resembling human rheumatoid arthritis, exhibiting a chronic, destructive and symmetrical infliction of the peripheral joints. Severe pannus formation and heavy destruction of cartilage and subchondral bone occurs in the acute phase within the first 2 weeks of the disease (Hoffmann et al, J Immunol 2007;179:7568–76), whereas little is known about the processes occurring in the later stages of the disease. The aim of this study was to perform an in-depth analysis of pathological changes occurring in bone and joints during the acute and chronic phase of PIA.
Materials and Methods: Male and female DA.1F rats were immunised intradermally with 150 µl pristane at the base of the tail and monitored for bone changes over a period of 130 days. Carpal joints, sacroiliac joints and vertebrae were analysed in vitro by histology as well as in vivo using micro-computed tomography with 20 µm resolution. The CatWalk system was used for automated gait analysis.

Results: Gait analysis identified gait changes such as reduced paw print areas in affected paws and altered step sequences approximately 2 days before the onset of clinically visual arthritis symptoms such as erythema and swelling. Whereas local inflammation and bone destruction occurred pronouncedly during the acute phase, extensive formation of new bone was the characteristic feature of the late stages of PIA. The formation of new bone led to an ossification of joints and rendered the animals permanently crippled. In the late stages of the disease ankylosis of the axial skeleton could also be observed.

Conclusion: Longitudinal studies of PIA employing micro-computed tomography and histological analyses showed that pristane-induced pathological processes appear to affect bone metabolism differently at different stages of the disease. These observations suggest that PIA cannot only serve as an experimental model of rheumatoid arthritis, but may also mirror the clinical picture seen in human ankylosing spondylitis.

A123 CHARACTERISATION OF JOINT PATHOLOGY IN IL-7 TRANSGENIC MICE: PILOT DATA
1E Horner, 1X Yang, 2F Fonchel. 1Oral Biology, LDI, Leeds University, Leeds, UK; 2Musculoskeletal Disease, LJM9, Leeds University, Leeds, UK

Background: IL-7, a member of the IL-2 family, has recently been associated with rheumatoid arthritis (RA) pathogenesis. It is expressed by stromal cells and activated dendritic cells (DC) and its receptor is expressed on all circulating T cells but not on B cells. IL-7 is essential for lymphopoiesis, T-cell development, DC1/Tly1 polarisation, T-cell survival and is produced in response to lymphopenia. RA synovial tissues and fluid show high levels of IL-7, and its expression is related to arthropathic measures of inflammation. These findings suggested that blocking IL-7 at the disease site could have therapeutic benefits. We have used an IL-7 transgenic mouse model to analyse directly the effects of increased expression of IL-7 in the joint in the absence of other confounding factors.

Methods: Both back legs from five transgenic mice and their wild-type littermates were dissected at the hip joint, taking care to keep the bone intact and fixed in formalin. Left legs were x rayed and the length and diameter of the femur, tibia and fibula were measured using callipers. Right legs were decalcified and paraffin embedded for histological analysis. Sections were stained using standard haematoxylin/eosin and fast green/Safranin “O” stains.

Results: Clear phenotypic differences were observed upon dissection between the transgenic mice and their wild-type littermates, with a greater femoral diameter noted in the transgenic mice. Following further de-fleshing and boiling, femurs appeared spongey with a greater femoral diameter noted in the transgenic mice. However, the arcuate lines and the shafts of bone in the transgenic mice were disorganised bone architecture and a possible outgrowth of the synovial membrane. In the knee we observed clear cartilage surface damage and outgrowth of the synovial membrane, with major inflammatory infiltrate in the transgenic mouse, suggestive of an RA-like phenotype.

Conclusion: These data are consistent with the hypothesis that elevated levels of IL-7 within the synovium may contribute to the onset of RA. Further investigations are, however, necessary to confirm that the cartilage defects observed are caused by the IL-7 phenotype and are not secondary to the bone phenotype.

A124 HO-1 END-PRODUCTS BILIVERDIN AND CARBON MONOXIDE REVEAL BENEFICIAL EFFECTS IN MURINE COLLAGEN-INDUCED ARTHRITIS
1M Bonelli, 1A Savitskaya, 1E Rath, 1F Bach, 1J Smolen, 1C Scheinecker, 1Division of Rheumatology, Internal Medicine III, Medical University of Vienna, Vienna, Austria; 2Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA

Introduction: Heme oxygenase 1 (HO-1), which degrades heme to free iron, biliverdin and carbon monoxide (CO), plays an important role in inflammation. It has already been shown that HO-1 also regulates osteoclastogenesis, which is driven by inflammatory cytokines. There are, however, conflicting data regarding the role of HO-1 in rheumatoid arthritis (RA) because treatment with tin protoporphrin IX, an inhibitor of HO-1, significantly reduced joint inflammation and cartilage destruction in the murine collagen-induced arthritis model (CIA). We therefore investigated the role of CO and biliverdin in the CIA mouse model.

Methods: CIA was induced in DBA/1 mice at week 12 of age. On day 0 mice were immunised with chicken collagen II (CII), emulsified in complete Freund’s adjuvant. After 14 days mice received a booster injection of CII. Mice were scored for paw swelling and grip strength. Anti-CII antibody levels were determined by ELISA. In addition, serum samples were analysed by flow cytometry multiplex kits for IFNγ, IL-6 and IL-17 on day 30. After the first clinical signs of arthritis one group of animals was treated with biliverdin (35 mg/kg) twice a day for 14 days. Bilirubin serum levels were measured 15 minutes post-intraperitoneal injection of biliverdin. The second group was treated for 14 days with CO (250 ppm) for one hour. After 50 days all animals were killed and paraffin sections of the affected joints were analysed for histomorphological signs of inflammation, cartilage and bone destruction.

Results: All animals immunised with CII developed serum anti-CII antibodies. Antibody levels were slightly decreased in biliverdin and, even more pronounced, in the CO-treated group. Animals in both the biliverdin and the CO-treated group showed an improvement in signs of clinical disease activity compared with control animals. In addition, decreased serum levels of IL-6, IL-17 and IFNγ were observed on day 30 in CO and biliverdin-treated animals. In line with the clinical data, histological analysis revealed less inflammation, erosion and reduced numbers of osteoclasts in both treatment groups.

Conclusion: Our data demonstrate a beneficial effect of HO-1 on inflammation and bone destruction in a murine model of RA.

A125 INHIBITION OF P38 EXACERBATES THE ONSET AND PROGRESSION OF SPONTANEOUS ARTHRITIS IN AGING MALE DBA/1 MICE, A MODEL OF SPONDYLOARTHRITIS
K Braem, I Derese, FP Luyten, RJU Lories. Laboratory for Skeletal Development and Joint Disorders, Katholieke Universiteit Leuven, Leuven, Belgium

Background: Spondyloarthritis (SpA) is characterised by chronic inflammation of the spine and joints as well as progressive ankylosis, leading to loss of function and disability. In spite of increasingly successful control of inflammation with tumour necrosis factor alpha-inhibitory agents, the structural progression of disease does not seem to be affected by these drugs. Bone
morphegenetic proteins (BMP) have been identified as key players in the onset and progression of ankylosis. BMP-associated intracellular signalling cascades (SMAD and p38 mitogen-activated kinase (MAPK) signalling) play a role during chondrogenesis, osteogenesis and inflammation in vitro and in vivo. We hypothesised that changes in the balance between SMAD and p38 signals can affect new tissue formation from the enthesis in the spontaneous mouse model of spondyloarthritides in DBA/1 mice.

Objectives: To study the effect of p38 MAPK inhibition on spontaneous arthritis in male DBA/1 mice.

Materials and Methods: Male DBA/1 mice from different litters were caged together at the age of 8 weeks (six males per cage). Two sets of experiments were performed. From week 10 onwards, mice were injected daily with SB203580 (50 μg/g body weight), a specific p38 inhibitor, or dimethyl sulfoxide (DMSO) (carrier) in a preventive strategy. Animals were killed at 17 weeks. Second, after the first symptoms appeared in each mouse, daily SB203580 (50 μg/g body weight) treatment was initiated and individual mice were killed 5 weeks after the start of treatment. The severity of disease was assessed twice a week by clinical scoring. At the end of the experiments hind paw forefeet were dissected and x-ray microtomography (μCT) and histomorphology were carried out to assess disease severity.

Results: Preventive treatment with the chemical inhibitor of p38 resulted in accelerated clinical incidence and higher severity scores compared with DMSO-treated controls. Pathology and μCT severity scores were also higher in the SB203580-treated group. The effect of p38 inhibition as a therapeutic strategy was evaluated after the first symptoms of arthritis appeared. SB203580 treatment significantly increased pathology severity score. Furthermore, incidence and μCT severity scores were also increased in the group receiving the p38 inhibitor. Observations of the different stages in the process of spontaneous arthritis suggest that p38 inhibition accelerates the progression of pathologic endothondral bone formation. Immunohistochemistry of phosphorylated SMAD1/5 and p38 revealed activation of these signalling pathways at different stages of ankylosing spondylitis.

Conclusion: These data suggest that inhibition of p38 exacerbates the development, severity and progression of spontaneous arthritis in male DBA/1 mice. We hypothesise that this may be due to an altered balance between BMP-induced SMAD in p38 MAPK signalling, favouring new tissue formation.

A126 LOSS OF P53 PARTLY RESCUES TUMOUR NECROSIS FACTOR-MEDIATED SYSTEMIC BONE LOSS

1S Hayer, 2M Hecking, 3DL Boyle, 4GS Firestein, 1G Schett. 1Medical University of Vienna, Internal Medicine II, Rheumatology, Vienna, Austria; 2Medical University of Vienna, Internal Medicine III, Nephrology, Vienna, Austria; 3Rheumatic Disease Core Center, Division of Rheumatology, Allergy and Immunology, University of California, San Diego, California, USA; 4University of Erlangen-Nuremberg, Internal Medicine 3, Erlangen, Germany

Objectives: To investigate the role of tumour suppressor protein p53 in tumour necrosis factor (TNF)-mediated arthritis in respect of inflammatory joint destruction and systemic bone loss.

Methods: To study the involvement of p53 in TNF-mediated arthritis, p53 knockout mice were crossed with human TNF transgenic (hTNFtg) mice. The clinical course of arthritis was assessed weekly from p53−/−hTNFtg and hTNFtg mice from 4 to 10 weeks of age. Paw sections from 10-week-old mice were histologically analysed for joint inflammation, subchondral bone erosion and cartilage damage using haematoxylin and eosin, TRAP and toluidine blue staining. To characterise osteoblast function, osteoblasts were isolated from 4-day-old calvarial bones and cultured in the presence of ascorbic acid and glycerol-2-phosphate for 20 days. Osteoblast differentiation and their capability for bone formation was evaluated by alizarin red staining, quantitative reverse transcriptase PCR and Western blotting. In addition, osteoblasts were counted each 3 days for 3 weeks to evaluate the growth rate of osteoblasts.

Results: p53 deficiency does not affect the clinical course of TNF-mediated arthritis. Progress of both paw swelling and grip strength was similar in p53−/−hTNFtg in comparison with hTNFtg mice from 4 to 10 weeks of age. Interestingly, loss of p53 results in reduced synovial inflammation, whereas subchondral bone erosion and cartilage damage did not differ between these two genotypes, as revealed by histological analysis of the hind paws of 10-week-old mice. However, histomorphological analysis of tibial bones revealed increased osteoblast formation and enhanced trabecular bone mass in p53−/−hTNFtg compared with hTNFtg mice. Interestingly, the loss of p53 demonstrates a dramatically enhanced proliferation in in-vitro osteoblast cultures from hTNFtg mice. Depletion of p53 leads to abolished expression of cyclin-dependent kinase inhibitor p21, as observed by Western blot analysis. Moreover, p53 deficiency demonstrated a higher osteoblast activity, as shown by increased bone nodule formation using alizarin red staining. Enhanced osteoblast differentiation demonstrated an increased expression of osteocalcin and bone sialoprotein messenger RNA as assessed by quantitative real-time PCR. Furthermore, increased osteoblast differentiation and bone nodule formation was accompanied by increased activation of MAP kinases p-Akt and p-ERK in p53−/−hTNFtg osteoblasts compared with hTNFtg osteoblasts, as shown by Western blots.

Conclusion: hTNFtg mice develop osteopenia as a result of enhanced osteoclastogenesis and reduced bone formation, respectively. The loss of p53 leads to increased activity of bone-forming osteoblasts and counteracts TNF-mediated inhibition of osteoblast-dependent bone formation. Depletion of p53 thus leads to more bone formation and can partly rescue TNF-mediated systemic bone loss.

A127 REGULATION OF INFLAMMATORY ARTHRITIS BY PTEN

1S Blu ¨ml, 2G Schabbauer, 1A Savitskaya, 1B Niederreiter, 1M Bonelli, 1J Smolen, 1K Redlich. 1Division of Rheumatology, Department of Internal Medicine III, Medical University Vienna, Vienna, Austria; 2Department of Vascular Biology and Thrombosis Research, Medical University Vienna, Vienna, Austria

Background and Objectives: Pten is a lipid phosphatase, whose substrate is phosphatidylinositol 3,4,5-trisphosphate. Therefore, pten is one of the main antagonists of PI3-kinase, which was shown to play a major role in many important cellular functions, such as proliferation, migration or response to inflammatory stimuli. Here we investigated the role of pten in the development of collagen-induced arthritis (CIA).

Methods: Analysis of monocyte/macrophage-specific pten knockout (LysMCrePtenflox/−) mice revealed significantly reduced joint inflammation, erosive bone destruction, as well as cartilage damage. Interestingly, total anticanine antibodies as well as anticanine IgG were identical in both groups. Analysis of cytokine levels in serum, however, showed significantly reduced production of IL-6, IL-8 as well as soluble E-selectin post-immunisation with collagen in LysMCrePtenflox/− mice. In vitro, induction of IL-6 in macrophages of pten-deficient mice was reduced in response to a variety of stimuli.

Conclusions: These data point to an important role of pten in arthritis development.
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ROLE OF THE CHOLINERGIC ANTI-INFLAMMATORY PATHWAY IN ARTHRITIS: AGGRAVATION OF ARTHRITIS IN ACETYLCHOLINE RECEPTOR ALPHA 7 SUBUNIT GENE KNOCKOUT MICE

1M van Maanen, 1,2 S Stod, 6 G LaRosa, 1,3 M Vervoordeldonk, 1,2 PP Tak
1Division of Clinical Immunology and Rheumatology, AMC/University of Amsterdam, Amsterdam, The Netherlands; 2Arthrogen BV, Amsterdam, The Netherlands; 3Critical Therapeutics, Inc, Lexington, Massachusetts, USA

Objective: The alpha 7 subunit of nicotinic acetylcholine receptors (α7nAChR) is abundantly expressed by immune cells and has been shown negatively to regulate the synthesis and release of tumour necrosis factor alpha (TNFα) by macrophages. We have previously shown in murine collagen-induced arthritis (CIA) that stimulation of α7nAChR by intraperitoneally injected AR-R17779, a compound that specifically binds to α7nAChR, can reduce the severity of arthritis, indicating that the α7 subunit plays an important role in synovial inflammation. In the present study, we investigated the effect of the absence of α7nAChR in CIA using α7-deficient (α7/-) and wild-type mice.

Methods: CIA was induced in α7/- and wild-type littermates (n = 20 per group) at day 0 by immunisation with chicken collagen type II (cCII) followed by a booster injection with cCII on day 20. Mice were killed on day 63 and clinical arthritis, radiological and histological damage was scored. In a separate study, mice were killed on day 44, the more acute phase of disease, and the effects on immune responses were evaluated by cytokine production and antigen-specific stimulation assays on spleen cells.

Results: In α7/- mice a 56% elevation in clinical arthritis scores and a significantly increased incidence of disease was observed. This was accompanied by an increase in bone degradation and synovial inflammation. Excacerbation of CIA was associated with elevated systemic proinflammatory cytokines and enhanced Th1-cytokine production by splenocytes.

Conclusions: The increase in severity and incidence of clinical arthritis in α7/- mice indicates that immune cell function is regulated by the cholinergic system and at least partly mediated by α7nAChR. The α7 subunit may be a future target for the treatment of rheumatoid arthritis.

A129

SPLENIC DENDRITIC CELL SUBSETS DURING COLLAGEN-INDUCED ARTHRITIS IN MICE: A ROLE FOR INFLAMMATORY CONVENTIONAL DENDRITIC CELLS?

1MC Lebre, 2L Bevaert, 3S Aarssen, 2J Koepke, 1PP Tak
1Division of Clinical Immunology/Rheumatology, AMC/University of Amsterdam, Amsterdam, The Netherlands; 2Arthrogen BV, Amsterdam, The Netherlands

Background and Objectives: Rheumatoid arthritis (RA) is an inflammatory autoimmune disease that leads to the destruction of synovial joints. In the synovial tissue there is massive accumulation of blood-borne cells that comprise activated T cells, B cells, plasma cells, macrophages and dendritic cells (DC). DC play a pivotal role in the orchestration of T-cell immunity and tolerance as a result of their ability to stimulate naive T cells and direct effector cell function. Most of the studies on DC in experimental arthritis have been conducted using (immunomodulated/tolerogenic) DC as tools to ameliorate experimental arthritis. Therefore, in order to gain insight into the characteristics of DC subsets in murine collagen-induced arthritis (CIA) we analysed the frequencies and phenotype of conventional (c)DC and plasmacytoid (p)DC in a murine C57BL/6 CIA model at different stages of the arthritis process.

Methods: Arthritis was induced in female C57Bl/6 mice on day 0 (intradermal injection of a chicken collagen/complete Freund’s adjuvant emulsion at the base of the tail) and on day 21 (intradermal injection with this emulsion was repeated). Mice were inspected daily from day 20 on for signs of arthritis by two independent observers. Clinical scores were assigned using an established macroscopic system. At different time points (at days 20, 30, 41 and 63 after CIA induction), mice were killed and spleens were collected. The frequency and phenotype of cDC and pDC was assessed by FACS using specific antibodies: CD11c (total cDC), CD8α (to distinguish two cDC populations: CD8α+ and CD8α–) and PDCA-1 together with B220 and Ly-6C (pDC). In addition, isolated cDC and pDC were stimulated for 48 h with lipopolysaccharide or CpG, respectively, and cell-free supernatants were analysed for the contents of inflammatory cytokines using a cytokine-bead assay.

Results: At all the time points studied, the frequencies of splenic cDC (total CD11c+, CD8α+ or CD8α–) significantly exceeded those of pDC (PDCA1 + B220 + Ly-6C+) except CD8α– at days 20 and 30. Within CIA mice the frequencies of CD8α– increased significantly (compared with day 20) starting from day 30, whereas the frequencies of CD8α+ and pDC were significantly increased on day 63 only. When all the DC subsets from CIA mice were compared with those present in mice without CIA the frequencies of total CD11c, CD8α+, CD8α– and pDC were significantly increased on day 63. Interestingly, within CIA mice activated cDC produced significantly higher levels of IL-6 on days 41 and 63, whereas tumour necrosis factor alpha (TNFα) was increased only on day 63 (compared with day 20). In contrast, IL-6 and TNFα derived from pDC decreased on day 63.

Conclusions: The observation that cDC subsets and their inflammatory cytokines are significantly increased during the development of CIA suggests an inflammatory role for these cells in the arthritic process. Achieving a detailed understanding of DC function(s) in CIA holds potential for modulating DC for immunotherapy by downregulating the autoimmune response.

A130

THE CARTILAGE-SPECIFIC MOLECULE, MELANOMA INHIBITORY ACTIVITY, CONTRIBUTES TO COLLAGEN-INDUCED ARTHRITIS

1Y Yeremenko, 1T Cantaert, 2A Bosserhoff, 1,2 PP Tak, 1D Baeten
1Division of Clinical Immunology and Rheumatology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands; 2Institute of Pathology, University of Regensburg, Regensburg, Germany

Objectives: Melanoma inhibitory activity (MIA) is a small chondrocyte-specific protein with unknown function. MIA-deficient mice have a normal phenotype and only minor microarchitectural alterations of the cartilage. We recently demonstrated that the immunodominant epitopes of MIA are actively presented in an HLA-DR4-restricted manner in the inflamed rheumatoid arthritis (RA) joint. To assess the potential role of MIA as an autoantigen, we investigated collagen-induced arthritis (CIA) in MIA-knockout mice.

Materials and Methods: 12-week-old wild-type C57BL/6 and MIA-knockout mice were immunised with chicken type II collagen in complete Freund’s adjuvant and boosted at day 21. The severity of arthritis was assessed by clinical scoring (0 to 16) and caliper. For flow cytometric analysis of lymphocyte phenotype, proliferation and cytokine production, splenocytes were collected at day 10 after either primary immunisation or boost and restimulation in vitro for 5 days with CD5/C2D28 (1 μg/ml/10 μg/ml), collagen type II (100 μg/ml), or MIA (100 μg/ml).

Results: MIA-knockout mice had a significant reduction in the clinical arthritis score in comparison with wild-type mice (p = 0.04). This resulted from both lower arthritis incidence (61.5% in MIA-knockout vs 72.2% in wild-type) and reduced arthritis severity in arthritic mice (mean maximum clinical score 6.8 ± 5.3 in MIA-knockout vs 9.2 ± 3.6 in wild-type). Accordingly, MIA-knockout mice exhibited a significantly reduced anti-collagen antibody level compared with wild-type controls (p = 0.02). Phenotyping of splenocytes showed higher percentages of CD8α+ CD44+ and CD4+ CD44+ cells in MIA-knockout mice.
of B cells (p = 0.03) and lower numbers of CD3+CD4− T cells (p = 0.01) in MIA-knockout mice. Ex-vivo restimulation with MIA of lymphocytes isolated after primary immunisation showed a significantly increased proliferation in wild-type versus MIA-knockout mice. Accordingly, there was a significant increase of Foxp3-expressing CD25+CD4+ regulatory T cells upon restimulation with not only MIA (p = 0.01) but also collagen (p = 0.02) in MIA-knockout mice. We did not find any difference in IFNγ production between wild-type and knockout mice. However, analysis of CD4 lymphocytes obtained after boost showed a significantly increased IL-17 production upon restimulation with collagen (p < 0.01).

**Conclusion:** MIA-deficient mice are partly protected against CIA. The inhibition of lymphocyte proliferation and the increase in regulatory T cells upon restimulation with MIA in vitro support a role for MIA as an autoantigen.

**A131** THE SECONDARY IMMUNE RESPONSE IS DEPENDENT ON THE NORMAL PRODUCTION OF REACTIVE OXYGEN SPECIES BY THE NADPH-OXIDASE COMPLEX

S Miranda, 1P Weinmann, 2PE Lipsky, 3R Holmdal, 4JE Fonseca, 5MM Souto-Carneiro, 1Rheumatology Research Unit, Instituto de Medicina Molecular, Lisbon, Portugal; 2NIAAS, National Institutes of Health, Bethesda, Maryland, USA; 3Medical Inflammation Research Unit, Karolinska Institute, Stockholm, Sweden; 4Centro de Neurocincias e Biomedicina, University of Comba, Comba, Portugal

Altered frequencies of circulating memory B cells have been associated with several autoimmune diseases, such as systemic lupus erythematosus, Sjogren’s syndrome, systemic sclerosis and rheumatoid arthritis. Recent data from chronic granulomatous disease patients—a primary immunodeficiency caused by deficient phagocytes to recurrent fungal and bacterial infections and the development of discoid lupus and chronic colitis—and their family members, suggest that absent or reduced production of reactive oxygen species (ROS) because of defects in the NADPH-oxidase complex (NOX2) are intimately linked to lower levels of peripheral blood memory B cells in humans.

Using mice with a point mutation in the Ncf1 gene leading to defective NOX2 function with consequent autoimmunity and an increased severity in collagen-induced arthritis, we studied a secondary immune response to ovalbumin to understand the role of ROS in memory B-cell development.

Wild-type B10.Q and Ncf1-defective B10.Q/Ncf1 (Ncf1∗) mice were immunised with ovalbumin plus alum (controls just received alum), challenged at day 40 and killed at day 45.

After the challenge, the Ncf1∗ mice had enlarged spleens, but with fewer germinal centres than wild-type mice. Moreover, splenic follicles in Ncf1∗ mice displayed an aberrant architecture characterised by a reduced presence of IgD+B220+ B cells within the follicle and an accumulation of IgD−IgM−B220+ B cells in the marginal zone. This was supported by flow cytometric data showing that Ncf1∗ spleens have significantly more marginal zone B cells (CD19+CD21+IgD+IgM−) than wild-type spleens. The titres of IgG anti-ovalbumin antibodies in response to secondary challenge were reduced in Ncf1∗ mice when compared with wild-type mice. Ncf1∗ mice also presented a lower frequency of circulating CD138+ plasma cells.

These results indicate that normal ROS production by NOX2 is essential for the generation of a secondary antibody response and for maintaining the integrity of the splenic architecture, and suggest that ROS play an essential role in the development of B-cell memory, thus challenging the dogma that ROS are solely pro-inflammatory and exacerbate inflammation, and opening new perspectives for novel therapies based on enhancing the oxidative capacity to control chronic inflammation.

**A132** CARTILAGE ENGINEERING USING PHARMACOLOGICALLY ACTIVE MICROCARRIERS COMBINED WITH MESENCHYMAL STEM CELLS

C Bouffi, 1O Thomas, 1C Bony, 1A Gitteau, 3M-C Verrier-Julienne, 1C Jorgensen, 1C Montero-Merie, 1D Nall, 1Inserm U844, Montpellier, France; 2Inserm U946, Angers, France

**Purpose:** Pharmacologically active microcarriers (PAM) are poly(D),l-lactide-co-lycolide) biocompatible and biodegradable microparticles, which may convey cells on their surface to provide an adequate three-dimensional microenvironment and deliver a growth factor to induce or maintain tissue-specific differentiation. Our objective was to implement PAM for tissue engineering of human mesenchymal stem cells (MSC) to stimulate chondrogenesis.

**Methods:** We set up a formulation of PAM enabling the continuous release of any desired growth factor from the microcarriers by developing a simple and reversible method to precipitate proteins and limit protein-polymer interactions for efficient encapsulation. To optimise the release kinetics, a biocompatible additive (poloxamer) was chosen using a protein/polymer adsorption model. The in-vitro release of transforming growth factor (TGF) beta3 from PAM was measured by ELISA and its activity was tested using a specific bioassay (Tesseur et al., 2006).

Chondrogenesis was induced by culture of 1.5 × 10⁵ MSC with 0.5 mg PAM in chondrogenic medium on low attachment culture plates for 21 days. Expression of the lineage-specific markers was quantified by reverse transcriptase quantitative PCR and immunohistochemistry using anticollagen 2 and anti-aggrecan antibodies on paraffin sections. For in-vivo experimentation, MSC were incubated for 24 h with PAM and injected (10 μl) into the knee joints of severe combined immunodeficient (SCID) mice for 5 weeks.

**Results:** The most efficient molecules for the biomimetic surface of PAM were fibronectin and poly-γ-lysine both for cell adhesion and survival of MSC in vitro. The in-vitro release of TGF-β3 reached 22% of the total amount of encapsulated protein by the first week and a plateau was observed after approximately 30% release by the first month. Importantly, more than 85% of released TGF-β3 was functionally active. When MSC were cultured in the presence of PAM-TGF, cells rapidly adhered onto PAM and progressively aggregated to form a unique pellet-like structure from day 7 to day 21. In PAM-TGF-induced aggregates, high expression of chondrogenic markers occurred in a time-dependent manner, whereas expression of osteogenic and adipogenic markers was lower than those observed when PAM coated with fibronectin were used. Intra-articular injection of MSC mixed with PAM-TGF confirmed their capacity to differentiate into cartilage tissue.

**Conclusion:** The combination of PAM with TGF-β3 allows MSC to differentiate preferentially into chondrocytes, both in vitro and in vivo. Indeed, PAM represent a promising strategy for delivering bioactive molecules that may be useful for tissue engineering.

**A133** MESENCHYMAL STROMAL CELLS IN SYNOVIAL FLUID OF CHILDREN WITH JUVENILE IDIOPATHIC ARTHRITIS AND CHILDREN WITHOUT JOINT INFLAMMATION

E Bernotiene, 1A Unguryte, 1V Panaviene, 1D Astraunike. 1Department of Experimental Research, Institute of Experimental and Clinical Medicine at Vilnius University, Vilnius, Lithuania; 2Clinic of Children’s Diseases, Faculty of Medicine, Vilnius University, Vilnius, Lithuania; 3Department of Rheumatology, Institute of Experimental and Clinical Medicine at Vilnius University, Vilnius, Lithuania

**Background and Objectives:** Mesenchymal stromal cells (MSC) are multipotent cells found in many adult tissues. As a result of their potential to give rise to skeletal cells as well as their...
immunosuppressive properties, MSC have attracted the attention of rheumatologists. Stromal cells in synovial tissue are increasingly studied, but the obtaining of synovial tissue samples is a complicated procedure, especially for children. Very few studies have investigated the presence of MSC in the synovial fluid (SF) of adults, reporting numbers of 2 colony forming units (cfu)/ml in rheumatoid arthritis, 37 cfu/ml in osteoarthritis and 14 cfu/ml in healthy controls (Jones et al., 2004; Jones et al., 2008), whereas data on SF-MSC in children are lacking. The aim of our study was to determine the presence and characteristics of SF-MSC in children with juvenile idiopathic arthritis (JIA) and children without inflammation in the joints.

**Materials and Methods:** 19 SF samples were obtained by puncture from 15 children, aged 5–17 years, including 12 patients with JIA (disease duration 1–41 months), and three children who were investigated for orthopedic pathology and served as controls. Cfu were established in relation to 106 total cells seeded and subsequently normalised to 1 ml of punctured SF. Multipotentiality at passage two was investigated by standard differentiation assays. The immunophenotype of cultured SF-MSC was determined by flow cytometry.

**Results:** SF-MSC were successfully isolated from all tested samples of SF of children either with JIA or controls without joint inflammation and displayed typical spindle-shaped morphology. On average, 74 cfu/ml and 118 cfu/106 of isolated cells were determined in SF in children with JIA and 31 cfu/ml and 139 cfu/106 of cells in controls, respectively. The differences are not significant as a result of high variation between the samples. Total punctured SF (mean 17 ml) generated on average 1229 cfu in children with JIA, and control SF (mean 2.5 ml) averaged 49 cfu, suggesting that the concentration of MSC resident in the joint does not decrease during joint swelling, but rather tends to accumulate. Similar to other types of MSC, stromal cells isolated from SF showed typical multilineage differentiation potential and expressed surface markers CD73, CD90, CD105, whereas for haematopoietic markers CD14, CD34 and CD45 were negative.

**Conclusions:** The presence of SF-MSC was determined in the SF of all tested children with JIA or without inflammation in the joints. In contrast to the published results in rheumatoid arthritis, numbers of SF-MSC were not reduced in children with JIA. SF could be an easily accessible source of joint-derived MSC for research and possibly diagnostic or therapeutic purposes.

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**Methods:** Two AAV plasmids coding for human IFNβ under the control of a nuclear factor kappa B promoter are generated. In order to reduce AAV-encapsidated residual DNA impurities, mostly arising from reverse packaging of the inverted terminal repeat, which are unwanted in clinical vector batches, we generated an “oversized” plasmid backbone (called “stuffer”), which exceeds the 4.7 kb natural packaging limit of AAV. In addition, we generated a conventional AAV plasmid containing a normal backbone size. Both plasmids were used for AA-V5 vector production, rAAV5.IFNβ.stuffer and rAAV5.IFNβ, respectively, and characterised in vitro. We first determined the DNA impurities and batch titre using a specific quantitative PCR. Thereafter, we transduced rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) with the recombinant AA-V5 vectors and stimulated with either tumour necrosis factor alpha (TNFα), IL-1β, or a combination of these cytokines. Levels of IFNβ and the effect on markers of inflammation (IL-6, IL-8, matrix metalloproteinase type 3; MMP-3) were determined by ELISA.

**Results:** As determined by quantitative PCR, both virus batches had a titre of 3.5 × 1011 viral particles per ml. Residual DNA analysis showed that the rAAV5.IFNβ.stuffer batch contained less than 1% impurity compared with 5% for rAAV5.IFNβ. Transduction of RA FLS with 2 × 1012 virus particles per ml showed similar human IFNβ production for both viruses. This resulted in a 50% (p < 0.05) decrease in pro-inflammatory cytokine production compared with the production in cells transduced with a control vector. Moreover, the levels of MMP-3 decreased, with more than 80% in cells transduced with both the rAAV5.IFNβ.stuffer and rAAV5.IFNβ vectors.

**Conclusion:** Transduction of RA FLS with both recombinant AA-V5 vectors resulted in a significant production of human IFNβ, which subsequently resulted in the reduced production of IL-6, IL-8 and MMP-3. However, as DNA impurities are a serious concern for the regulatory authorities we selected the rAAV5.IFNβ.stuffer vector for clinical application because batches of this vector showed a reduced impurity level. The gene construct will now be produced under good manufacturing practice conditions and undergo extensive quality assurance/quality control testing for toxicology studies, an important step towards clinical application of the vector.
with infliximab. The reversion of arthritis was followed by a late flare in TNFK-treated animals; a late boost of TNFK (17 weeks after priming) allowed a regression of arthritis in comparison with non-boosted animals. The histological score evaluated 30 weeks after priming showed the persistence of the anti-inflammatory effects, although a slight progression was noticed in TNFK-treated animals between the 12th and 30th week after vaccination. Anti-TNFα antibodies decreased along time after a peak approximately 8 weeks after TNFK priming.

Conclusions: TNFK vaccination is efficient in treating established arthritis in TTg mice. The blockade of TNFα induced by TNFK immunisation is reversible, as demonstrated by clinical, histological and TNFα level evaluation and may be re-induced by a late boost of TNFK. Taken together, these data are consistent with a unique profile of this anti-TNFα vaccination strategy, with a clear-cut long-lasting effect on chronic inflammation contrasting with a reversible active blockade of TNFα.

**A136** LIPOSOMAL TARGETING OF GLUCOCORTICOID TO MACROPHAGES INHIBITS OSTEOCLASTOGENESIS AND BONE EROSION

1W Hofkens, 1B Walgreen, 2G Storm, 3W van den Berg, 1P van Lent. 1Rheumatology Research and Advanced Therapeutics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; 2Department of Pharmaceutics, Utrecht University, Utrecht, The Netherlands

Background and Objectives: Bone resorption in inflamed joints is a common pathological feature during rheumatoid arthritis. This process is principally mediated by osteoclasts, which secrete bone resorbing enzymes such as tartrate-resistant acidic phosphate (TRAP) and cathepsin K. Osteoclasts differentiate out of the monocyte/macrophage lineage under the influence of macrophage-colony stimulating factor (M-CSF) and receptor activator of the nuclear factor kappa B kinase ligand (RANKL). In an inflamed joint, macrophages can be targeted with liposomes containing anti-inflammatory glucocorticoids and thereby influence the differentiation towards osteoclasts or mature osteoclasts and inhibit bone erosion. The objective of this study was to study the inhibitory effect of liposomal glucocorticoid on osteoclastogenesis and bone erosion.

Materials and Methods: Mice with established antigen-induced arthritis were treated with a single intravenous injection of 10 mg/kg liposomal or free prednisolone phosphate (PLP) or control injections (phosphate-buffered saline (PBS) or empty liposomes). After 8 days, the mice were killed for histology of the knee joints. Inflammation on the synovium and bone erosion were scored from frontal knee joint sections on an arbitrary scale between 0 and 3. Cathepsin K expression was determined by immunostaining. Cytokine levels were determined from synovial wash-outs with Luminex multi-analyte technology. Mouse bone marrow cells were differentiated into osteoclasts by stimulation with M-CSF and RANKL for 7 days and were visualised with TRAP staining. Bone resorption was determined after Coomassie blue staining of resorption pits after culturing osteoclasts on rat bone slices. The uptake of rhodamine–phycocerythrin-labelled liposomes was determined by flow cytometry.

Results: A single injection of liposomal PLP resulted in the inhibition of joint inflammation by 91% and bone erosion by 46% from PBS-treated mice and was significantly more potent than an inhibition of joint inflammation by 91% and bone erosion by 46% for bone erosion compared with PBS treatment). The actions of liposomal PLP were reflected in a 75% reduction in cathepsin K staining in the knee joint. Liposomal targeting of bone-marrow-derived macrophages in vitro resulted in a complete inhibition of differentiation towards osteoclasts, as determined by TRAP-positive staining and the presence of multinucleated cells. Bone resorption by osteoclasts in vitro was inhibited by liposomal glucocorticoid when liposomes were added during differentiation, but not when liposomal glucocorticoids were added during the last 2 days when osteoclasts had fully matured. Moreover, during the differentiation towards osteoclasts, bone narrow macrophages show a much lower uptake of fluorescent liposomes, suggesting that liposomal glucocorticoids inhibit osteoclastogenesis rather than mature osteoclasts.

Conclusion: Liposomal glucocorticoids inhibit bone erosion by targeting macrophages and inhibiting their differentiation towards osteoclasts.

**A137** NEW INSIGHTS INTO NON-GENOMIC EFFECTS OF GLUCOCORTICOSTEROIDS: HOW METHYLPREDNISOLONE SUCCINATE CAN CREATE A STATE OF PSEUDO-HYPOXIA AND INDUCE APOPTOSIS

1P Remans, 1D Gerlag, 2S Houten, 3L Nijtmans, 2J Ruiter, 1B Leerkotte, 1PP Tak. 1Division of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; 2Department of Clinical Chemistry, Laboratory of Genetic Metabolic Diseases, Emma Children’s Hospital, Amsterdam, The Netherlands; 3Department of Paediatrics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Background: Methylprednisolone succinate (MPS) pulse therapy (1 g for 3 days) is commonly used to treat severe manifestations of autoimmune diseases. We have previously shown that treatment of peripheral blood mononuclear cells (PBMC) with MPS induces apoptosis. This is regulated by Nip3, a pro-apoptotic molecule known to regulate hypoxia-induced apoptosis.

Objective: To investigate the molecular mechanism leading to MPS-induced apoptosis.

Materials: Oxygen consumption of purified mitochondria was measured with an oxygraph. For adenosine triphosphate (ATP) production, PBMC were permeabilised with digitonin and treated with different substrates of the oxidative phosphorylation (ox phos) process, including malate, glutamate and adenosine 5'-diphosphate. ATP was subsequently measured by fluorometry. Blue native gel electrophoresis and spectrometric in-gel analysis were used to separate the different ox phos complexes and to determine their individual enzyme activity. For aconitase and citrate synthetase activity an adapted spectrophotometric assay was used. Western blots were used to determine Nip3 and HIF-1α expression and apoptosis was measured with annexin/propidium iodide staining.

Results: MPS induced a concentration-dependent inhibition of the ox phos process at concentrations greater than 50 μmol, as measured by the inhibition of mitochondrial oxygen consumption and a reduction in ATP production. MPS specifically inhibited complex I and complex V. No inhibition of complexes II, III or IV was observed. Inhibition of the ox phos was followed by inhibition of the Krebs cycle, which was determined by consumption of (5-H3) and (6-H3) glucose. Inhibition of the ox phos was also accompanied by the rapid production of mitochondrial free radicals. Aconitase is the mitochondrial enzyme that catalyses the isomerisation of citrate to isocitrate in the Krebs cycle, and is known to be highly sensitive to oxidative stress and free radicals. In our experiments, inhibition of the ox phos and the generation of mitochondrial free radicals was accompanied by a profound decrease of aconitase enzyme activity (but not citrate synthetase), which explained why we measured an increase in pyruvate but a strong decrease in alpha-ketoglutarate (AKG). AKG is a substrate of prolylhydroxylase, which regulates the ubiquination of HIF-1α and the deprivation of AKG results in stabilisation of HIF-1α and adaptation to anaerobic metabolism. We detected HIF-1α after treatment with MPS greater than 100 μmol after 24 h and Nip3 expression and subsequent apoptosis after 48 h. In addition, cells with oxidative stress were sensitised to MPS-induced apoptosis and apoptosis was observed greater than 10 μmol.
Conclusions: MPS specifically inhibited ox phos complexes I and V, which resulted in the complete inhibition of the Krebs cycle and generation of free radicals. This pseudohypoxic state induced apoptosis via HIF-1 and Nip3. Inflammatory cells, which are highly metabolically active and have oxidative stress, are sensitised to MPS-induced apoptosis. MPS-induced apoptosis may help explain the effectiveness of MPS pulse therapy.

A139 | PROTEIN BIOCHIP ARRAY TECHNOLOGY TO MONITOR RITUXIMAB IN RHEUMATOID ARTHRITIS

1 S Fabre, 1 C Guisset, 1 T Tatem, 1 N Dossat, 1 AM Dupuy, 1 JP Cristol, 1 JP Daures, 1 C Jorgensen, 1 Immuno-rheumatology, Lapeyronie University Hospital, Montpellier, France; 2 Biochemistry Laboratory, Lapeyronie University Hospital, Montpellier, France; 3 Institut Universitaire de Recherche Clinique, Montpellier, France.

Background and Objectives: In rheumatoid arthritis (RA) there are currently no good indicators to predict a clinical response to rituximab. The purpose of this study was to monitor and determine the role of peripheral blood cytokine profiling in differentiating between a good versus a poor response to rituximab in RA.

Materials and Methods: Blood samples were collected at baseline and at 3 months from 46 RA patients who were treated with rituximab. Responders are defined by the presence of three out of four American College of Rheumatology criteria: 20% or greater decrease in C-reactive protein (CRP), visual analogical score of disease activity, erythrocyte sedimentation rate and improvement of the disease activity score (DAS28, four values) by 1.2 or greater obtained at 3 months. Twelve cytokines were measured from serum collected on days 0 and 90 by proteomic array (Randox, France) including IL-6, tumour necrosis factor alpha, IL-1a, IL-1b, IL-2, IL-8, IFNγ, IL-4, IL-10, monocyte chemotactic protein type 1 (MCP-1), epidermal growth factor (EGF) and vascular endothelial growth factor.

Results: We showed that CRF and IL-6 levels decrease significantly at 3 months in the responder group compared with baseline. We identified at day 90 a cytokine profile that differentiates responders and non-responders. High serum levels of two pro-inflammatory cytokines, MCP-1 and EGF, were significantly higher in the responder group at day 90 compared with non-responders. However, we were not able to identify a baseline cytokine profile predictive of a good response at 3 months.

Conclusions: These findings suggest that cytokine profiling by proteomic analysis may be a promising tool for monitoring rituximab and may help in the future to identify responder RA patients.

A140 | STRATEGY TO IDENTIFY A MICRO RNA SIGNATURE OF MESENCHYMAL STEM CELLS ACQUIRING A STABLE CHONDROCYTE PHENOTYPE

1 JG Godeladze, 2 D Noel, 2 M Brodello, 2 H Yssel, 2 U Nuber, 2 F Djouadi, 2 C Bony, 2 G Courlies, 3 J Presumay, 1 R Richard, 2 F Apparailly, 1 C Legall, 1 C Jorgensen. Department of Biochemistry, IMBS, University of Oslo, Oslo, Norway; 2Inserm U844, Montpellier, France; 3University of Lund, Lund, Sweden; 4IBM CNRS, Montpellier, France.

Background and Objectives: Cell engineering for cartilage replacement necessitates knowledge of the mechanisms for the differentiation of chondrocytes from stem cells (eg, mesenchymal stem cells; MSC), as well as an insight into mechanisms ascertaining the stability of this phenotype. The present work summarises one strategy to identify a group of micro RNA, the presence of which may secure acquisition of the chondrocytic phenotype.

Methods and Results: First, small interfering RNA against Drosha and Dicer were used to identify the point in time when the differentiation of chondrocytes was most sensitive to perturbation of the miRNA synthesising machinery. Second, transcription modulators (TM; such as Runx2, Osterix, SFP, TAZ, Satb2, ATF4, NFATc1, RNF11, HES1, LIF, ETS1, APC, VDR and others) important for the pre-commitment and development of osteoblastic cells from stem cells, were identified (through a literature search). Then, various databases (MirNAViewer, PicTAR, Sanger) were consulted for common miRNA species putatively targeting these TM. 20 miRNA species were obtained, simultaneously targeting some three to five TM. Of these, 10–15 miRNA proved to be
vastly absent in osteoblasts and highly expressed in chondrocytes (shown by differential display miRNA microarray; LC Science, USA; and quantitative PCR performed on single samples) on day 3 of differentiation. Subsequently, the possible targeting of early upregulated genes in chondrocytes (as determined from a literature search and the GenoStem expression array with ~60 000 genes) was checked. Of the said 10–15 miRNA species, six miRNA were selected for further studies, including in-vitro differentiation, re- and de-differentiation using pre-miRNA, antago-miRNA and anti-sense miRNA oligonucleotides. Selected parameters for chondrogenesis (eg, marker gene expression, histology, immunohistochemistry, GAG/DNA contents and clinical scores of micropellets) were analysed in parallel. Then, the six miRNA species were analysed for synergistic effects on osteoblast suppression, and for possible “boosting” of chondrocyte differentiation using pre-miRNA and antago-miRNA and marker gene expression. Furthermore, the putative targets were identified using PsicHECK-based (Promega, Madison, WI, USA) luciferase reporter constructs, in which relevant parts of their 3’UTR-region were amplified. This experiment enabled ranging of the various miRNA species in terms of their regulatory potential. Finally, chondrocytes differentiated from MSC, while being transfected with pre-miRNA related to the six said miRNA for up to 14 days in culture, were able to maintain their phenotype (assessed by marker gene expression and impact on osteoclastogenesis), when challenged with culture media containing tumour necrosis factor alpha and interleukins known to be associated with inflammation.

Conclusion: This strategy selecting miRNA species, suppressing the pre-commitment and differentiation of osteoblasts, has proved successful in maintaining the phenotype of differentiating chondrocytes derived from MSC.

**Results:** In fibroblast-like synoviocytes TNFα-induced iNOS and MMP-13 messenger RNA expression was significantly (p<0.05) and dose-dependently inhibited by pFTY. Furthermore, synoviocyte but not chondrocyte proliferation was significantly enhanced by co-treatment with TNF-α and pFTY. In chondrocytes IL-1β and TNFα-induced iNOS and MMP-13 transcription was significantly inhibited by pFTY. These results were confirmed by Western blot. Furthermore, IL-1β-induced cartilage degradation was inhibited by pFTY720 in a significant and dose-dependent manner by up to 54.7% at a concentration of 6 μmol.

**Conclusions:** Our results suggest that FTY7720 in contrast to S1P reduces cytokine-induced transcription of MMP-13 and iNOS in synovial tissue and cartilage. Adding these new findings and its known immunosuppressive properties, FTY7720 could be a useful therapeutic option in rheumatoid arthritis.

The first two authors are joint first authors.

**Background and Objectives:** Whereas current biotherapies have improved rheumatoid arthritis (RA) treatment, they still have drawbacks, supporting the need for the development of alternative strategies. Transforming growth factor beta (TGFβ)-activated kinase 1 (TAK1), a downstream mediator of TGFβ, IL-1β and tumour necrosis factor alpha (TNFα) signal pathways, plays a central role in the regulation of inflammation and tissue destruction in the context of diseases such as RA. TAK1 may thus be considered an attractive drug target for inflammatory arthritis. We propose to investigate the feasibility of targeting the TAK1-mediated cascades in vivo by RNA interference in an experimental model of arthritis using an innovative therapeutic approach.

**Methods:** TAK1 small interfering RNA sequences were validated in vitro on the macrophage cell line J774.1 at both the messenger RNA and protein levels, and downregulation of downstream p38 and JNK-activation was assessed after TNF challenge. For in-vivo administration, 10 μg of siRNA were formulated as lipoplexes with the RPR209120/DOPE liposome and a carrier DNA, and injected intravenously into DBA/1 mice having collagen-induced arthritis (CIA). The clinical course of the disease was assessed by paw thickness over time and histological scores were obtained at the time of killing (tartrate-resistant acid phosphate (TRAP) activity, inflammation and erosion area). The immunological balance was assessed using anti-type II collagen (bII) assays, measuring the bII-specific T-cell proliferation, quantifying cytokine levels in sera and knee-conditioned media by ELISA, and determining the T regulatory and Th17 cell numbers in sera, liver and spleen. The cellular mechanisms of lipoplex action were investigated more specifically using fluorescent microscopy and FACs analysis of fluorescent siRNA and immune cell surface markers.

**Results:** The TAK1 siRNA sequence reproducibly silenced at least 50% of the protein expression compared with a control siRNA, as well as the phosphorylation of the downstream JNK signalling pathway. In the CIA model, weekly intravenous injections of anti-TAK1 siRNA lipoplexes significantly reduced the severity of established arthritis, compared with the control siRNA lipoplex-injected group. The clinical effect was associated with a decreased secretion of IL-6, IFNγ and TNFα both locally (knee joints) and systemically (blood and spleen), as well as with a reduction of structural changes in joints and modification in the numbers of...
IFNγ/IL-10-producing cells in the liver. There was no effect on the antigen-specific induced T and B-cell responses and no change in the number of IL-17-producing lymphocytes. The siRNA formulation mainly targets macrophages within the liver and spleen.

**Conclusion:** TAK1 being implicated in pathways regulating inflammation, synovial proliferation and bone homeostasis appears to be an interesting novel therapeutic target, blockade of which efficiently combines a reduction of inflammation and protection of joint structure in RA.

**Methods:** Bone marrow-derived MSC expansion medium (n = 6 donors) was modified by replacing human FCS with plasma and platelet extract. Cells were characterised according to the defined minimal criteria for multipotent MSC by analysing their surface antigen expression and differentiation capacity. FCS-free differentiation was performed in six-well plates, replacing FCS with different concentrations of human plasma from several donors (n > 3). After 21 days of incubation in adipogenic or osteogenic induction medium, cells were analysed by cytological staining. Furthermore, messenger RNA expression of the adipogenic markers, ppARG2 and LPL, and the osteogenic markers, Runx2 and osteocalcin, were investigated. In addition, chondrogenic markers (aggrecan, type II collagen) were analysed to exclude spontaneous chondrogenic differentiation.

**Results:** Adipogenic and osteogenic differentiation of FCS-free expanded MSC in FCS-free induction medium yielded cells with adipogenic and osteogenic phenotypes as well as characteristic gene expression. In adipocytes the lipid vesicles were marked with oil red O staining and characterised by mRNA expression of ppARG2 and LPL. Osteogenic differentiation revealed calcified spots positive for von Kossa staining as well as mRNA expression levels of osteopontin and osteocalcin. The results do not indicate any spontaneous chondrogenic differentiations.

**Conclusion:** Human plasma is a suitable FCS replacement for adipogenic and osteogenic differentiation and therefore represents a feasible strategy for tissue engineering under GMP-compatible protocols.