**Autoantibodies and autoantigens**

1

**Differential antibody recognition of the 349–364aa B-cell epitope of human La/SSB protein and its phosphorylated analogue**

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**Background** La/SSB is a phosphoprotein that associates with various small RNA molecules. It has been found that the primary phosphorylation site of the molecule during various physiological processes is in Ser366.

**Objectives** To determine whether the phosphorylation state of Ser366 could affect the antigenicity and the recognition of the protein by antibodies from patients with primary Sjögren’s syndrome (pSS).

**Methods** Peptides 349–368aa and phos349–368aa (with the Ser366 residue phosphorylated) were synthesized. Sera with anti-La specificity from 30 patients with pSS and sera from 19 normal individuals were examined against the two synthetic peptides in ELISA. The antibody specificity against the epitopes was tested with homologous and heterologous inhibition assays.

**Results** Of pSS sera 23% reacted against the 349–368aa peptide. Sera binding to unphosphorylated peptide reacted also with phos349–368aa. Although the same sera gave a positive reaction against both peptides, the optical density values received from antibodies to phos349–368aa were higher, indicating a higher concentration or stronger affinity. When phos349–368aa was used as soluble inhibitor, in homologous inhibition the reactivity was almost completely abolished (92%). In contrast, when the unphosphorylated peptide was used as inhibitor, the reactivity of sera against phos349–368aa was only partially reduced (35%), indicating that sera from these patients possess two distinct groups of antibodies: one against the unphosphorylated epitope and one against the phosphorylated epitope.

**Conclusion** The phosphorylation of the serine366 residue resulted in a significant increase in antibody binding on epitope 349–368aa of La/SSB. These observations might explain the increased antigenicity of La/SSB autoantigen in various pathological situations in which phosphorylation may occur.

2

**Surface-bound immune complexes containing antibodies to collagen type II induce production of TNF-α, IL-1β and IL-8 from monocytes via FcγRII**

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**Background** Antibodies to collagen type II (anti-CII) are found in a subpopulation of rheumatoid arthritis (RA) patients. In joint inflammation CII epitopes are exposed to anti-CII, with possible formation of surface-bound immune complexes (IC). We investigated whether surface-bound CII anti-CII IC can induce cytokine production from mononuclear cells and the mechanisms behind this.

**Methods** ELISA plates were coated with native human CII and blocked with human serum albumin, after which sera with varying concentrations of anti-CII were added. Serum-free mononuclear cell cultures from healthy blood donors were then added, and after 20 hours cytokine levels in supernatants were measured using ELISA. Parallel wells without cell cultures were developed as anti-CII ELISAs. Sixty-five RA patients and 10 control individuals were investigated cross-sectionally, and 17 patients with anti-CII followed longitudinally for 1–5 years. FcγRII and FcγRIII were blocked with specific antibodies. Cell depletion/enrichment studies were performed to define responder cells. High sensitivity CRP measurement was performed with nephelometry.

**Results** Surface-bound IC containing CII induce TNF-α, IL-1β and IL-8 from mononuclear cells via FcγRII. Cytokine production correlated highly with anti-CII levels in the cross-sectional investigation. Five out of the six longitudinally followed patients with highest anti-CII levels also showed parallel changes in anti-CII OD, cytokine induction and CRP. Depletion/enrichment studies showed monocytes to be the responding cells.

**Conclusion** Surface-bound anti-CII IC can form in inflamed joints. Such IC can induce proinflammatory cytokines such as TNF-α, IL-1β and IL-8 from monocytes via FcγRII. Most serially followed patients with high anti-CII levels showed parallel changes in anti-CII, induced cytokines and CRP. This may imply mechanisms of pathophysiological importance in the subpopulation of RA patients with high levels of anti-CII.

3

**Prothrombin fragment F1+2 in patients with antiphospholipid antibodies**

A Ambrozic, B Bozic, S Cucnik, T Kveder, B Rozman

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**Background** Studies of specific markers for *in vivo* activation of coagulation in patients with antiphospholipid antibodies (aPL) are very rare.
Increased levels of prothrombin fragment F1+2 (F1+2) in patients with APS were reported. **Objective** Our aim was to ascertain the relationship of F1+2 plasma levels with positive anticardiolipin (aCL) and anti-β2-glycoprotein I (anti-β2-GPI), and to evaluate the effect of treatment on F1+2 values in patients with APS. **Methods** A total of 205 samples from 177 patients with suspected or confirmed connective tissue disease without APS, and 15 samples from nine patients with APS receiving anticoagulant (n = 8) or antiplatelet (n = 1) therapy were tested for plasma F1+2 values (Enzygost F1+2 micro, Behring, Germany). aCL (IgG, IgM) and anti-β2-GPI (IgG, IgM, IgA), all using in-house ELISAs. **Results** Elevated values of F1+2 were statistically significantly associated with medium/high positive results for at least one isotype of aCL (P = 0.007), anti-β2-GPI (P = 0.0019) and aCL and/or anti-β2-GPI (P = 0.014; Table 1). Furthermore, the mean level of F1+2 was significantly higher in patients with medium/high aCL or anti-β2-GPI than in those with negative/low positive aCL and anti-β2-GPI (P = 0.035). In all 15 plasma samples from APS patients, normal levels of F1+2 were measured during treatment. **Table 1**

<table>
<thead>
<tr>
<th>Association of F1+2 with aCL and/or β2-GPI</th>
<th>Normal F1+2</th>
<th>Elevated F1+2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCL and β2-GPI negative/low positive</td>
<td>128 (81%)</td>
<td>31 (19%)</td>
<td>159</td>
</tr>
<tr>
<td>aCL and/or β2-GPI medium/high positive</td>
<td>29 (63%)</td>
<td>17 (37%)</td>
<td>46</td>
</tr>
</tbody>
</table>

**Conclusions** Our study showed a significant association of aCL and anti-β2-GPI with elevated levels of F1+2 in patients without APS not receiving anticoagulant or antiaggregation therapy. aPL are believed to be among the important causes of hypercoagulable state in those patients. Furthermore, plasma values of F1+2 could be a very useful indicator of successful treatment in APS patients.

**5 Concomitant appearance of anti-cardiolipin, anti-β2-glycoprotein I, anti-prothrombin and anti-annexin V antibodies**

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**Background** Anticardiolipin (aCL), anti-β2-glycoprotein I (anti-β2-GPI), anti-prothrombin (aPT) and anti-annexin V (aANXV) antibodies are antiphospholipid antibodies (aPL) with different antigenic and diagnostic specificities.

**Objective** To ascertain a simultaneous appearance of antigenically undefined aCL and antigenically distinct anti-β2-GPI, aPT and aANXV.

**Methods** Sera from 92 patients (87 female, five male) were studied (63 SLE, 19 sAPS [SLE with APS] and 10 pAPS). IgG, IgM and IgA isoforms of aPL were determined using relevant ELISAs.

**Results** IgG was the most frequently detected isotype in all four analyzed aPL subtypes. According to diagnostic criteria, aCL were more frequently found in patients with pAPS and sAPS than in those with SLE. Similarly, aPT were more often elevated in patients with pAPS (71/10) and sAPS (8/19) than in SLE (16/63; P < 0.05). The prevalence of anti-β2-GPI and aANXV antibodies did not differ between patient groups. Regardless of diagnosis, aPT were always detected in combination with aCL and/or anti-β2-GPI. aPT and aANXV were regularly present in the sera positive also for aCL and/or anti-β2-GPI. aANXV was detected as a single antibody in only one patient. In the pAPS group concurrent multiple aPL varieties (three or four) were significantly more frequently present than one or two types (8/10 versus 2/10; P < 0.05). Statistically significant associations of particular aPL with clinical symptoms were as follows: elevated IgG anti-β2-GPI with arterial thromboses, thrombocytopenia and foetal loss; IgG aPT with arterial thromboses; and IgG aCL with venous thrombosis. IgA isotype did not improve the clinical significance of the four analyzed aPL.

**Conclusion** In patients with SLE and/or APS, individual aPL were found infrequently. Almost half of them had three or more aPL subsets elevated at the same time.

**6 Association of anti-ribosomal P protein antibodies with neuropsychiatric systemic lupus erythematosus**

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**Background** There is an unclear association of anti-P with neuropsychiatric (NP) SLE.

**Objective** Our objective was to determine the prevalence of anti-P in 150 SLE patients, focusing on psychosis and/or mood disorders, other diffuse NP syndromes and focal NP syndromes, and to analyze the manifestation of NP syndromes regarding SLE duration and anti-P status.

**Methods** One hundred and fifty Caucasian SLE patients (133 female, 17 male) were studied, 88 of whom had a disease duration of more than 5 years. IgG anti-P were detected by immunoblotting performed on ribosomal extract of HeLa cells and/or by line immunoassay (INNO LIA ANA). NP manifestations presenting at any time were defined according to ACR case definitions for NP SLE. Patients in whom...
diffuse and focal NP manifestations occurred at the same time were classified according to the predominant manifestation.

**Results** Anti-P were positive in 17/150 (11.3%) patients. Diffuse NP manifestations were diagnosed in 9/17 (53%) anti-P positive patients (Table 1). Statistically significant associations of anti-P were found with (1) psychosis and/or mood disorders (13/11, P<0.05), (2) other diffuse NP syndromes (6/14, P<0.0001), and (3) all diffuse NP syndromes (9/25; P<0.0001), as compared with patients without diffuse NP manifestations (7/92). In the group of 88 patients with disease duration greater than 5 years, an even stronger statistically significant association of anti-P with diffuse NP manifestations was found.

**Table 1**

<table>
<thead>
<tr>
<th>Study findings</th>
<th>Anti-P positive</th>
<th>Anti-P negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n = 17 )</td>
<td>( n = 133 )</td>
</tr>
<tr>
<td>1. Psychosis and/or mood disorders</td>
<td>3 (18%)</td>
<td>8 (6%)</td>
</tr>
<tr>
<td>2. Other diffuse NP syndromes</td>
<td>6 (35%)</td>
<td>8 (6%)</td>
</tr>
<tr>
<td>3. Only focal NP syndromes</td>
<td>1 (6%)</td>
<td>32 (24%)</td>
</tr>
<tr>
<td>4. Without NP manifestations</td>
<td>1 (41%)</td>
<td>85 (64%)</td>
</tr>
</tbody>
</table>

**Conclusion** In our study anti-P antibodies showed a strong association with diffuse but not focal NP syndromes in SLE patients, especially those with disease duration over 5 years.

7 Human cartilage glycoprotein 39-directed T cell responses in health and arthritic diseases

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**Objective** Although (self)antigen-directed T cells are thought to be key mediators of many autoimmune diseases, a functionally distinct population of CD4+ T cells – T regulatory cells (Treg cells) – dominantly inhibit induction and progression of autoimmunity, as demonstrated in several autoimmune models. In humans the presence of Treg cells has been shown, but their role in autoimmune disease is not known. Likewise, no (auto)antigens recognized by Treg cells have yet been identified at the molecular level. The aim of these studies was to gain a better understanding of the identity of (auto)antigens recognized by Treg cells.

**Results** When analyzing the natural T cell response against a candidate autoantigen in rheumatoid arthritis (RA), namely human cartilage gp-39 (HC gp-39), we found that healthy donors, although displaying a typical Th1 reaction against a mixture of recall antigens, reacted against HC gp-39 by producing IL-10. The IL-10 production was mediated by CD4+ T cells. When HC gp-39-directed immunity of RA patients was analyzed, a marked contrast was observed as Th1-like reactivity was observed in a substantial number of patients as determined by the production of IFN-γ. More importantly, we found that HC gp-39-directed immunity in healthy donors inhibits the T cell response against a mixture of recall antigens. Likewise, HC gp-39-specific immunity as well as HC gp-39-directed, IL-10-producing CD4+ T cell lines were able to suppress MHC class I-restricted CTL reactivity.

**Conclusion** Together, these data point to a disease-associated bias in the type of T cell response against HC gp-39, and identify HC gp-39 as a naturally occurring autoantigen that is recognized by Treg cells in humans.

8 Multiplex analysis of antinuclear antibodies by flow cytometry using FIDIS

PJ Charlies

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**Background** FIDIS (BMD, France) is a multiplex analytical flow cytometry system for the detection of antibodies. The aim of this study was to evaluate the FIDIS connective assay system for the detection of antinuclear antibodies (ANA), and to assess the clinical utility of these parameters in the diagnosis of connective tissue diseases. The FIDIS system simultaneously measures IgG antibodies directed at dsDNA, Ro, La, RNP, Sm, Jo-1, Scl-70, rRNP, and CENP-B.

**Methods** Sera were obtained from 100 patients with SLE and 100 patients with Sjögren’s syndrome. A total of 78 sera from patients with rheumatoid arthritis (RA) and 100 from normal healthy blood donors were also tested.

**Results** Antibodies to Scl-70, Jo-1, and rRNP were not detected in any of the sera from the disease groups. In the normal control group, the only positive result obtained was in one sera that gave a weak positive result for anti-CENP-B.

**Table 1**

<table>
<thead>
<tr>
<th>Frequency of antibodies detected using FIDIS™</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>-----</td>
</tr>
<tr>
<td>Anti-Ro</td>
</tr>
<tr>
<td>Anti-La</td>
</tr>
<tr>
<td>Anti-Sm</td>
</tr>
<tr>
<td>Anti-RNP/Sm</td>
</tr>
<tr>
<td>Anti-CENP-B</td>
</tr>
<tr>
<td>Anti-dsDNA</td>
</tr>
</tbody>
</table>

**Conclusion** The FIDIS connective assay system simultaneously measures IgG antibodies directed against nine different specificities. The data presented indicate that this system provides an alternative strategy for the measurement of specific ANA to the use of multiple single analyte assays currently employed in many immunology laboratories.

9 Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice

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The current view holds that chronic autoimmune diseases are driven by the continuous activation of autoreactive B and T lymphocytes. However, despite the use of potent immnosuppressive drugs designed to interfere with this activation, the production of these autoantibodies often persists and contributes to progression of the immunopathology. In the present study, we analyzed the lifespan of (auto)antibody-secreting cells (ASC) in the spleens of NZB/W mice, a murine model of human systemic lupus erythematosus (SLE). The number of splenic ASC increased in mice aged 1–5 months and...
became stable thereafter. Less than 60% of the splenic antibody-secreting cells were short-lived plasmablasts, whereas 40% were non-dividing, long-lived plasma cells with a half-life of more than 6 months. In NZB/W mice and D42 immunoglobulin heavy chain ‘knock-in’ mice, we found that a fraction of DNA-specific plasma cells were also long-lived. Although antiproliferative immunosuppressive therapy eliminates short-lived plasmablasts, long-lived plasma cells can survive and continue to produce (auto)antibodies. Thus, long-lived, autoreactive plasma cells are a relevant target for researchers aiming to develop curative therapies for autoimmune diseases.

Acknowledgement Both senior authors (RAM and FH) contributed equally to this work.

10 Immune complexes from RA patients induce FcγRII-dependent and RF-correlated TNF-α and IL-8 production from healthy PBMC

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Background Immune complexes (IC) induce production of cytokines from mononuclear cells via Fcγ receptors. We investigated whether polyethylene glycol (PEG)-precipitated IC from serum and synovial fluid (SF) from rheumatoid arthritis (RA) patients can induce production of proinflammatory cytokines.

Methods In one study we compared sera and SF from 15 RA patients and 15 healthy controls. In a second study we used paired sera and SF from 32 RA patients, 66% of which were rheumatoid factor (RF) positive. The precipitates where diluted to the original volume in PBS before 10% were added to serum-free PBMC cultures from two healthy blood donors. After 20 hours TNF-α and IL-8 levels were measured using ELISA. In separate cell culture experiments FcγRII and FcγRIII were blocked. RF in serum was determined by nephelometry and IgG levels in precipitates were measured using ELISA.

Results We found a correlation between TNF-α induced by PEG precipitates from RA SF and RF levels in sera. Using the normal ELISA, PEG precipitates were shown to contain some TNF-α but no IL-8, whereas the expression of CXCR3 on plasma cells, thus suggesting that the regulation of this receptor is an early event during plasma cell differentiation. In contrast, CXCR4 was present on all plasma cells, formed under any stimulation conditions and even when CXCR4-negative B cells were activated T dependently in a two-step culture system, for 3 days by CD40 ligand together with IL-2 and IL-10 and for 5 more days with IL-2 and IL-10. Alternatively, cells were stimulated T independently with CpG, IL-2 and IL-10. Addition of IFN-γ or monocytic/T-cell culture supernatant in the initial culture, but not at late stages, induced the expression of CXCR3 on plasma cells, thus supporting the hypothesis that the regulation of this receptor is an early event during plasma cell differentiation. The expression of CXCR4 and, as a consequence, the potential to migrate into the bone marrow is generally associated with differentiation into plasma cells, whereas the expression of CXCR3 must be induced by the inflammatory cytokine IFN-γ.

11 Differential regulation of the expression of chemokine receptor 3 and 4 during plasma cell differentiation

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The duration of specific antibody titers ranges from a few weeks up to several years. While the formation of antibody secreting plasma cells takes place in secondary lymphoid tissues, long-lasting antibody responses are provided by bone marrow plasma cells. The majority of plasma cells initially formed remains in secondary lymphoid tissues and die within a few days. Plasma cells that migrate into the bone marrow or into chronically inflamed tissues can survive for much longer periods of time. The capacity of plasma blasts to migrate into these tissues is regulated by chemokine receptors. Expression of CXCR4 is important for plasma cell migration into the bone marrow. CXCR3 ligands expressed in large quantities in chronically inflamed tissues (e.g. in affected kidneys in systemic lupus erythematosus) and can attract plasmablasts, thus mediating plasma cell accumulation at these sites. Here we analyzed the regulation of chemokine receptor expression during plasma cell differentiation in culture. Purified CXCR3-negative B cells were activated T dependently in a two-step culture system, for 3 days by CD40 ligand together with IL-2 and IL-10 and for 5 more days with IL-2 and IL-10. Alternatively, cells were stimulated T independently with CpG, IL-2 and IL-10. Addition of IFN-γ or monocytic/T-cell culture supernatant in the initial culture, but not at late stages, induced the expression of CXCR3 on plasma cells, thus suggesting that the regulation of this receptor is an early event during plasma cell differentiation. In contrast, CXCR4 was present on all plasma cells, formed under any stimulation conditions and even when CXCR4-negative B cells were activated to form plasma cells. These data indicate that expression of CXCR4 and, as a consequence, the potential to migrate into the bone marrow is generally associated with differentiation into plasma cells, whereas the expression of CXCR3 must be induced by the inflammatory cytokine IFN-γ.

12 Two B cell populations differentially expressing IgVH mRNAs in human RA synovium

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1Institute of Rheumatology and LGE, Prague, Czech Republic; 2Department of Immunology and Gnotobiology, CAS, Prague, Czech Republic; 3Department of Rheumatology, Charité, Berlin, Germany


Background The lymphocytic infiltrates sometimes organized in ectopic germinal centres in rheumatoid arthritis (RA) synovium contain locally activated B cells expressing hypermutated immunoglobulin transcripts and recombination-activating genes (Rag), suggesting an ongoing antigen driven process directly in synovium.

Objective To test this hypothesis, mutual frequencies of immunoglobulin mRNAs, signs of isotype switching and Rag gene expression in individual RA synovial B cells were analyzed.

Methods Single-cell RT-PCR was used to analyze individual synovial CD19+CD38+ and CD19+IgM+ B cells (as the reference population) from two RA patients.

Results We found significantly reduced frequencies of peripheral blood CD19+CD38+ B cells from RA patients as compared with controls, suggesting their possible migration into the site of inflammation. Three subsets of CD19+CD38+ B cells in RA synovium were detected, expressing (1) only IgM transcripts (IgM+, 13.5%), (2) only IgG tran-
scripts (IgG+, 48.7%), and (3) both IgM and IgG mRNAs (IgM+IgG+, 37.8%). The differences in mutational frequencies between them were significant (Table 1). Over 40% of analyzed cells coexpressed Rag mRNAs. Similar subsets and expression patterns were found in CD19+IgM+ B cells; however, IgG+ cells displayed significantly decreased mutational frequencies (3.6%; P<0.0001). This might reflect the presence of synovial B cell populations that differ in their maturational status or biological function.

Table 1

<p>| Mutational frequencies of IgVH mRNAs in RA synovial CD19+CD38+ and CD19+IgM+ populations |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>IgM+</th>
<th>IgM+IgG+</th>
<th>IgG+</th>
<th>IgM+</th>
<th>IgM+IgG+</th>
<th>IgG+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total of nt</td>
<td>1761</td>
<td>6617</td>
<td>4683</td>
<td>2128</td>
<td>2570</td>
</tr>
<tr>
<td>Mutated nt</td>
<td>94</td>
<td>465</td>
<td>462</td>
<td>75</td>
<td>110</td>
</tr>
<tr>
<td>MF (%)</td>
<td>5.3</td>
<td>7.0</td>
<td>9.9</td>
<td>3.5</td>
<td>4.3</td>
</tr>
</tbody>
</table>

13 Immunomics in inflammatory rheumatic diseases

Autoimmune diseases such as rheumatoid arthritis (RA) are characterized by autoantibodies to different autoantigenic proteins. Using proteomic 2D immunobLOTS, we identified a new 40 kDa autoantigen – hnRNP A3 – from HeLa nuclear extracts, which is frequently (30%) detected by RA. Moreover, we used a set of protein arrays of about 50,000 proteins derived from a human foetal brain cDNA expression library for screening with patient sera. Additionally, we utilized a human foetal brain cDNA library in a robot-based T7 phage display screening system with RA patient sera. To determine the diversity of the enriched library, we amplified the cDNA inserts and hybridized them onto the custom-made human ENSEMBL cDNA array. By these methods, over 80 clones were identified to bind patient immunoglobulins. Moreover, nine clones show only IgA-specific reactivity. We have now evaluated two different clones thoroughly: the carboxyl-terminal half of the nuclear phosphoprotein p130 (NOPP 130) and a clone representing a 41-amino-acid mimetic peptide showing homology to calreticulin, previously reported as autoantigen. The remaining proteins are still undergoing thorough investigation. Applying state-of-the-art proteomic techniques, such as protein array and phage display, we have succeeded in identifying more than 80 potentially autoantigenic marker molecules, of which we have characterized a subset for RA specificity by screening with large numbers of patient and control sera. However, none of the molecules characterized thus far is exclusively discriminatory for RA and they all exhibit overlap with other autoimmune diseases.

Autoantibodies: anticitrullin antibodies

14 VIDAS-EDRA fully automated testing of autoantibodies to citrullinated proteins for the diagnosis of rheumatoid arthritis

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Antiperinuclear factor and antikeratin antibodies (AKA) were shown to belong to the same family of rheumatoid arthritis (RA)-specific autoantibodies directed at ‘citrullinated’ peptidic epitopes borne by (pro)flaggrins. We showed that their major target in the synovial tissue of RA patients is deiminated (citrullinated) fibrin. Although (pro)flaggrins are probably only cross-reactive proteins, their in vitro detection allowed the development of several highly efficient tests for the diagnosis of RA. Among those, the CCP ELISAs (CCP1 and CCP2), based on a cyclic citrullinated peptide derived from human flaggrin, and the ARFA-ELISA that we developed using a citrullinated recombinant rat flaggrin appear to be the most promising. The rapidly growing interest of rheumatologists in autoantibodies to citrullinated proteins rendered the development of a convenient, fully automated test the next challenge. Based on the ARFA-ELISA, we developed an automated test on the VIDAS machine (bioMérieux), called VIDAS-EDRA (early diagnosis rheumatoid arthritis). Thresholds were defined on a large series of 617 patients with well defined, established rheumatic diseases, including 181 patients with RA. Antibodies to CCP1 and CCP2 (Immunoscan, Eurodiagnostica) were sought following the manufacturer procedures. Rheumatoid factor (RF) and AKA were also analyzed in the series. The diagnostic performances of the tests were compared. In established diseases the diagnostic sensitivity of the VIDAS-EDRA was found to be identical to that of CCP2, and was significantly higher than tests for AKA, CCP1 and RF. Autoantibodies to citrullinated proteins can be efficiently detected with the highly specific and sensitive automated test VIDAS-EDRA.

Table 1

<table>
<thead>
<tr>
<th>Diagnostic sensitivity (%) computed at thresholds allowing 95.2% and 98.6% diagnostic specificity to be achieved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>AKA</td>
</tr>
<tr>
<td>RF</td>
</tr>
<tr>
<td>CCP1</td>
</tr>
<tr>
<td>CCP2</td>
</tr>
<tr>
<td>VIDAS-EDRA</td>
</tr>
</tbody>
</table>

15 Features of the citrullinating peptidylarginine deiminase enzymes

ER Vossenaar, AJW Zenden, R Rajmakers, TJ Welting, A van der Heijden, WAM Horstman, K Cheung, HG Vogelzangs, S Nijenhuis, WJ van Venrooij, GJM Pruijn

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Background Autoantibodies to citrullinated proteins (anti-CCP) can be detected in up to 80% of rheumatoid arthritis (RA) patients with very
high specificity (98%). Citrulline residues, the target of the anti-CCP antibodies, are formed by post-translational modification of arginine residues, catalyzed by peptidylarginine deiminase enzymes (PAD; EC 3.5.3.15).

**Objective** Our aim was to investigate the full complexity of the family of PAD enzymes and to locate putatively important conserved residues or domains.

**Methods** We performed a thorough query for PAD sequences using EST and genomic databases. With these data we constructed a complete PAD alignment (CustalW) and a phylogenetic tree (Treeview), and mapped the different genes.

**Results** Next to the previously described PADs, we found several (partial) novel sequences, both mammalian and nonmammalian. From the alignment (available online at http://www.interscience.wiley.com/jpages/0265-9247/suppmat/2003/25/v25.1106.html), it is clear that the carboxyl-terminal half is more conserved than the amino-terminal part. Some of the fully conserved residues (His475 and Cys655) have been suggested to be important for catalytic activity of murine PAD2. These residues would fit in the "catalytic pocket," as has been described for other arginine converting enzymes (arginine deiminases EC 3.5.3.6. and amidotransferases EC 2.1.4.1.). The negative charge (on average –14) and low pI (about 5.8) of the enzyme are important for its interactions with arginine substrates and with Ca2+ ions, which are essential for PAD activity. A phylogenetic tree confirmed the human PADs to be the orthologue of the murine PAD4. By comparing mRNA and genomic sequences, the individual exons of all murine and human PADs could be mapped in a tight conserved gene cluster (human chromosome 1p36.1; mouse #4E1; rat #5q36). Structural characterization of PADs will yield valuable clues regarding the aetiology of RA and for the development of PAD inhibiting drugs.

### 16 False positivity of rheumatoid factor and antibodies to citrullinated peptides in systemic lupus erythematosus

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**Background** Rheumatoid factor (RF) is found in patients with systemic lupus erythematosus (SLE). Anti-citrullinated peptide antibodies (ACPA) are more specific for rheumatoid arthritis than RF.

**Objective** Our aim was to determine the prevalence of RF and ACPA in SLE patients.

**Methods** In this study, samples from 201 consecutive patients diagnosed with SLE and fulfilling ACR criteria were used. Fine ANA reactivities were tested by INNO-LIA®ANA (Innogenetics, Gent, Belgium) and by IIF on C. luciliae. RF was detected by latex fixation. ACPA were detected by anti-CCP2 ELISA (Euro-Diagnostica, Arnhem, The Netherlands) and by a research INNO-LIA®RA (Innogenetics, Gent, Belgium) for the detection of anti-pepA and anti-pepB antibodies. The prevalences of ACPA were compared by the McNemar test.

**Results** RF at a titre ≥160 was found in 26 patients (13.0%). ACPA were found in 16 samples (Table 1). The prevalence of anti-CCP2 antibodies was significantly higher than that of anti-pepA antibodies ($P = 0.001$) and anti-pepB antibodies ($P = 0.022$).

**Conclusion** RF is found in 13.0% of SLE patients. Anti-CCP2 antibodies are false-positive in 7.0% ($n = 14$) of SLE patients, which occurs significantly more often than anti-pepA (1.5%, $n = 9$) and anti-pepB (2.5%, $n = 5$) antibodies.

### Table 1

<table>
<thead>
<tr>
<th>Clinical</th>
<th>Anti-CCP2 (titre)</th>
<th>Anti-pepA</th>
<th>Anti-pepB</th>
<th>ANA fine reactivities</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF</td>
<td>1280</td>
<td>+</td>
<td>+</td>
<td>Ro60, SSB</td>
</tr>
<tr>
<td>0</td>
<td>28</td>
<td>–</td>
<td>–</td>
<td>SmB, SmD, histones, dsDNA</td>
</tr>
<tr>
<td>640</td>
<td>9</td>
<td>–</td>
<td>+</td>
<td>RNP-C</td>
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<tr>
<td>0</td>
<td>168</td>
<td>–</td>
<td>–</td>
<td>dsDNA</td>
</tr>
<tr>
<td>0</td>
<td>53</td>
<td>–</td>
<td>–</td>
<td>SmB, dsDNA</td>
</tr>
<tr>
<td>80</td>
<td>2</td>
<td>–</td>
<td>+</td>
<td>Histones, dsDNA</td>
</tr>
<tr>
<td>0</td>
<td>76</td>
<td>–</td>
<td>–</td>
<td>SmB, RNP-A, RNP-C, RiboP, histones</td>
</tr>
<tr>
<td>40</td>
<td>64</td>
<td>–</td>
<td>–</td>
<td>SmD, SmB, RNP-70k, RNP-C, RiboP</td>
</tr>
<tr>
<td>0</td>
<td>58</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>320</td>
<td>110</td>
<td>–</td>
<td>–</td>
<td>SmB, RNP-70k, RNP-A, RNP-C, Histones, dsDNA</td>
</tr>
<tr>
<td>0</td>
<td>28</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0</td>
<td>36</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>320</td>
<td>78</td>
<td>+</td>
<td>+</td>
<td>SmB, RNP-70k, RNP-A, RNP-C, RiboP, Histones</td>
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<tr>
<td>80</td>
<td>56</td>
<td>–</td>
<td>–</td>
<td>RNP-A</td>
</tr>
<tr>
<td>640</td>
<td>52</td>
<td>–</td>
<td>–</td>
<td>RNP-70k, RNP-A</td>
</tr>
<tr>
<td>320</td>
<td>1600</td>
<td>+</td>
<td>+</td>
<td>Ro60</td>
</tr>
</tbody>
</table>

### 17 Diagnostic performance and predictive value of serum markers for the diagnosis of rheumatoid arthritis

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**Background** Rheumatoid factor (RF) is the classical serum marker for rheumatoid arthritis (RA). Anti-citrullinated peptide antibodies (ACPA) have a higher sensitivity and specificity (spec) for RA.

**Objective** Our aim was to evaluate the diagnostic and predictive value for RA of RF and antibodies to pepA and pepB (two synthetic substrates for ACPA detection) in a setting relevant to routine clinical practice.

**Methods** In this prospective multicentre study, samples were collected at academic and nonacademic centres from 1003 consecutive patients presenting for diagnostic work-up when the clinician included RA in the differential diagnosis. RF was detected by latex fixation. A research INNO-LIA®RA (Innogenetics, Belgium) was used to detect anti-pepA and anti-pepB antibodies. Diagnoses were made by the clinician using ACR criteria after 1 year follow-up. ROC curve analysis was used to evaluate the diagnostic performance of the tests.
The aim of this study was to compare the recognition patterns of antibodies in rheumatoid arthritis (RA) patients. The presence of these markers early in disease, even before clinical onset, and the observation that these autoantibodies are produced locally in the pannus suggest an involvement in the pathogenesis. The targeted epitopes are generated by deimination, a post-translational modification catalyzed by the enzyme peptidyl arginine deiminase.

Objective Our aim was to analyze the presence of Cit-proteins and fibrinogen in the joints at different time points in collagen-induced arthritis (CIA) in rats and to investigate how citrullination of an autoantigen affects its immunogenicity.

Methods Synovial tissue sections from DA rats were stained for expression of citrulline and fibrinogen. Lew1AV1 rats were immunized with Cit-rat serum albumin (RSA) or unmodified RSA, and antibody and T-cell responses were evaluated.

Results Citrulline was detected in arthritic joints from disease onset and increased expression was noted as disease progressed into a more chronic state. Naïve rats or time points before arthritis onset were negative for citrulline-specific staining. Infiltrating cells, as well as the cartilage surface, stained positive for citrulline, although the major source of citrullinated proteins appeared to be fibrin depositions. A specific Cit-RSA T-cell response was observed in animals challenged by Cit-RSA. In contrast, no response was recorded when RSA was used as a stimulus. The IgG response revealed not only a response toward the modified protein but also cross-reactivity to the unmodified form of RSA.

Conclusion In CIA, joint inflammation precedes the presence of Cit-proteins and citrullination increases immunogenicity of an autoantigen. Our results suggest that citrullination is induced by inflammation and might be contributing to the development of autoreactive T and B cells.

19

Antifilaggrin antibodies in serum and synovial fluid samples of patients with rheumatoid arthritis show similar reactivity pattern towards citrulline containing peptides

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Background Antifilaggrin antibodies are highly specific serological markers of rheumatoid arthritis (RA). They have been shown to comprise a heterogeneous population of antibodies directed at citrullinated peptides. Recent studies suggest that the site of the initial antigenic trigger where these autoantibodies are produced can be localized to the synovial tissue.

Objective The aim of this study was to compare the recognition patterns of antibodies in paired serum and synovial fluid samples of RA patients toward citrullinated peptide sequences to investigate whether or not they comprise the same antibody population.

Methods Arginine-rich peptide sequence corresponding to human profilaggrin (amino acid residues 306–324) and sequences with citrulline substitution at different positions were synthesized by mutipin peptide synthesis on solid support. Completely citrullinated variant of the 19-mer peptide and shortened sequences were also produced. The reactivity of these peptides with paired sera and synovial fluid samples of RA patients were determined (n = 25). Results were evaluated statistically using the paired t test.

Results and Conclusion The results (Table 1) show that the 12–19 amino acid long epitopes are recognized by homogeneous antibody population present in serum and synovial fluid, whereas the reactivities toward short citrullinated sequences differs significantly.

Acknowledgement This work was supported by the Hungarian grant: OTKA T037876.

Table 1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cutoff</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
</tr>
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<tbody>
<tr>
<td>PepA</td>
<td>Low</td>
<td>63.6</td>
<td>90.6</td>
<td>60.7</td>
</tr>
<tr>
<td>PepB</td>
<td></td>
<td>64.3</td>
<td>90.0</td>
<td>59.0</td>
</tr>
<tr>
<td>RF</td>
<td></td>
<td>69.2</td>
<td>90.1</td>
<td>61.5</td>
</tr>
<tr>
<td>PepA</td>
<td>Intermediate</td>
<td>62.9</td>
<td>95.1</td>
<td>74.4</td>
</tr>
<tr>
<td>PepB</td>
<td></td>
<td>61.5</td>
<td>95.1</td>
<td>73.3</td>
</tr>
<tr>
<td>RF</td>
<td></td>
<td>55.2</td>
<td>94.6</td>
<td>69.9</td>
</tr>
<tr>
<td>PepA</td>
<td>High</td>
<td>58.7</td>
<td>98.1</td>
<td>87.5</td>
</tr>
<tr>
<td>PepB</td>
<td></td>
<td>48.3</td>
<td>98.1</td>
<td>85.2</td>
</tr>
<tr>
<td>RF</td>
<td></td>
<td>42.0</td>
<td>97.8</td>
<td>81.1</td>
</tr>
<tr>
<td>PepA</td>
<td>Very high</td>
<td>41.3</td>
<td>99.0</td>
<td>90.8</td>
</tr>
<tr>
<td>PepB</td>
<td></td>
<td>37.1</td>
<td>99.0</td>
<td>89.8</td>
</tr>
<tr>
<td>RF</td>
<td></td>
<td>21.0</td>
<td>99.0</td>
<td>83.3</td>
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</tbody>
</table>

Conclusion When high specificity is required, anti-pepA and anti-pepB antibodies have a markedly higher sensitivity than RF. The highest PPV are found when ACPA are very high.

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Citrullinated proteins in arthritis: presence in joints and effects on immunogenicity

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1Department of Medicine, Karolinska Institutet, Stockholm, Sweden; 2Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands


Background Autoantibodies (Ab) directed against citrulline (Cit)-containing proteins have a specificity of nearly 100% in rheumatoid arthritis (RA) patients. The presence of these markers early in disease, even before clinical onset, and the observation that these autoantibodies are produced locally in the pannus suggest an involvement in the pathogenesis. The targeted epitopes are generated by deimination, a post-translational modification catalyzed by the enzyme peptidyl arginine deiminase.

Objective Our aim was to analyze the presence of Cit-proteins and fibrinogen in the joints at different time points in collagen-induced arthritis (CIA) in rats and to investigate how citrullination of an autoantigen affects its immunogenicity.

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19

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M Brózik1, A Magyar2, R Tobi3, G Bálint1, ZS Balogh1, A Polgár1, F Hudecz2, K Merényi1

1National Institute of Rheumatology, Budapest, Hungary; 2Peptide Chemistry Research Group, Eötvös Loránd University, Academy of Science, Budapest, Hungary


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Acknowledgement This work was supported by the Hungarian grant: OTKA T037876.
The presence of deiminated fibrin in the synovial membrane is not specific for rheumatoid arthritis

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Background Autoantibodies to deiminated (citrullinated) forms of the α and β chains of fibrin (AhFibA), also known as anti-filaggrin autoantibodies (AFA), are the most specific serological markers of rheumatoid arthritis (RA). Deimination is critical in generating the AhFibA epitopes and, in the synovial tissue (ST), deiminated fibrin is their major antigenic target. Existence of fibrin deimination specifically in RA patients could explain why the AhFibA response is RA specific.

Methods and Results To explore such an association, the presence of deiminated fibrin in the ST was assessed in a series of 32 patients, 13 with RA and 19 with non-RA rheumatological disorders (controls). ST biopsies were collected in macroscopically inflamed areas identified under needle arthroscopy of the knee. Histopathological examination confirmed the existence of synovitis in all of the samples. The presence of deiminated fibrin was first assessed by immunoblotting of ST extracts using antibodies to citrullyl residues and to the α and β chains of fibrin, and AhFibA purified from a pool of RA sera. Deiminated fibrin was evidenced in all of the ST samples. Moreover, variations in the ratio of deiminated to total fibrin were not related to diagnosis. Immunohistochemical analysis, performed on adjacent synovial biopsy sections of 19 of the 32 patients, using antibodies to the β chain of fibrin and to citrullyl residues, allowed us to confirm the results obtained by immunoblotting, because deiminated fibrin was detected in six RA patients and six controls.

Conclusion Our results show the presence of deiminated fibrin in the ST is not specific for RA and suggest that it is induced by ST inflammation. Moreover, they show the production of AhFibA is not a direct consequence of the presence of deiminated fibrin in the ST. Nonetheless, AhFibA are tightly associated with RA. Because they are also present very early in the disease course, linked to disease severity and produced in the ST, they probably play an important role in RA pathophysiology.

Cytokines and chemokines

Elevated expression of IL-18 in Sjögren’s syndrome: distinct expression patterns of the active and proactive forms

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Department of Pathophysiology, School of Medicine, National University of Athens, Athens, Greece


Background IL-18 is an important immunoregulatory and proinflammatory cytokine that is produced by various cell types, including antigen-presenting cells and epithelial cells. IL-18 is synthesized as a biologically inactive precursor molecule (proactive IL-18) that converts into an active, mature molecule after enzymatic cleavage. Upregulated levels of IL-18 have been identified in several autoimmune diseases, but its role in Sjögren’s syndrome (SS) is uncertain.

Objective The aim of the study was to investigate the role of IL-18 in SS.

Methods RT-PCR analysis, immunohistochemistry and immunoblotting (with distinct anti-IL-18 antibodies against the proactive and active forms) were applied for the detection of IL-18 in cultured SG epithelial cell (SGEC) lines established from patients with SS (n = 5) and from non-SS disease control patients (n = 5), as well as in minor SG biopsy specimens obtained from patients with primary SS (n = 12), secondary SS (n = 5) and non-SS disease controls (n = 7) were used.
Results The expression of IL-18 mRNA was detected in all SGEC lines tested. In addition, cultured SGEC express constitutively the proactive, but not the mature, form of IL-18 protein, as demonstrated by immunoblotting. In minor SG biopsies, strong expression of the IL-18 proactive protein was detected in the ductal epithelial and CD68+ infiltrating macrophages of the SS patients, but not in the controls. The expression of the active form of IL-18 was detected only in SS patients and mainly in those with extended inflammatory lesions. In these samples, active IL-18-positive cells were observed exclusively among the CD68+ macrophages of the mononuclear cell infiltrates.

Conclusion Our data possibly indicate the role of IL-18 in the expansion of lymphoepithelial lesions of SS patients. SGEC were found to express only the proactive form of IL-18, the biological significance of which remains to be determined.

23 Cytokine imbalance in NZB/W mice is associated with autoantibody levels and nephritis P Enghard1, D Langnickel1, F Hiepe1,2, GR Burmester1, G Riemekasten1
1Department of Rheumatology, Charité University Hospital, Berlin, Germany; 2German Rheumatology Research Center, Berlin, Germany Arthritis Res Ther 2004 6(Suppl 1):23 (DOI 10.1186/ar1065)

Background In the NZB/W murine model of lupus, various studies have analyzed cytokine production during disease development with contradictory results.

Objective The aim of this study was to analyze cytokine pattern of splenic T cells from NZB/W mice. We assessed the possibility that a shift in cytokine production is associated with age, disease activity, or manifestation of nephritis.

Methods NZB/W mice of different ages spanning 5–36 weeks were analyzed, and healthy (BALB/c × NZW) F1 mice were used as controls. Proteinuria was measured using albumin. Plasma creatinine and autoantibody levels were detected by commercial test kit and by ELISA, respectively. Splenic CD4+ T cells stimulated with PMA/Ionomycin were analyzed for intracellular cytokine staining by FACS analysis.

Results The increased frequency of IFN-γ producing CD4+ T cells correlated with age, anti-dsDNA IgG antibody levels and proteinuria. The increasing number of IL-10 producers was only associated with anti-dsDNA antibody levels and proteinuria. A small gain in IL-4+ T cells correlated with plasma creatinine. Neither the number of IL-10 nor that of IL-4-expressing T cells correlated with age. There was no significant change observed in the production of TNF-α. Calculating the IFN-γ/IL-4 ratio, an increasing shift toward a Th1 response was observed. The shift strongly correlated with anti-dsDNA antibody titres and proteinuria. In contrast, no cytokine shift could be observed in control mice with increasing age.

24 Anti-tumour necrosis factor therapy modulates the OPG/OPGL system in rheumatoid arthritis synovium AI Catrina1, S Ernestam2, E af Klint1, L Klareskog1, AK Ulfgren1
1Rheumatology Department, Karolinska Institute/Hospital; 2Rheumatology Department, Huddinge Hospital, Stockholm, Sweden Arthritis Res Ther 2004 6(Suppl 1):24 (DOI 10.1186/ar1066)

Background Osteoprotegerin (OPG) is a soluble decoy receptor that acts as a receptor antagonist, blocking OPG ligand (OPGL)-induced osteoclastogenesis. In vitro, proinflammatory cytokines such as tumour necrosis factor (TNF) upregulate endothelial OPGL expression. However, low levels of endothelial OPGL expression are seen in synovial biopsies from patients with active rheumatoid arthritis (RA) associated with high levels of TNF. In order to elucidate the in vivo interaction between TNF and the OPG/OPGL system in RA, we investigated the effect of anti-TNF therapy on synovial expression of OPG and OPGL.

Methods OPG, OPGL and CD31 were evaluated by immunohistochemistry in serial synovial biopsies obtained from 20 RA patients before and after 8 weeks of treatment with Etanercept (Amgen and Wyeth Pharmaceuticals, USA) or Infliximab (Scherering-Plough, Stockholm, Sweden). Biopsies were evaluated by double-blind semiquantitative analysis and image analysis. Statistical analysis was performed using the Wilcoxon’s signed-rank test followed by Bonferroni corrections for multiple comparisons.

Results OPG was expressed in all studied synovial biopsies, mainly in the CD31-positive endothelial cells. OPGL expression was detected in synovial areas rich in T cells, with low expression in endothelial cells and macrophages. Treatment with Infliximab significantly increased synovial OPGL expression whereas the increase in synovial OPGL expression observed after Etanercept treatment did not reach statistical significance. Neither infliximab nor etanercept influenced OPGL expression following 8 weeks of treatment. The ratio between synovial OPGL and OPGL expression decreased following treatment with infliximab (P<0.05, after Bonferroni comparisons) and etanercept (NS after Bonferroni correction).

Conclusion Our findings suggest a particular OPG–TNF interaction in vivo as well as a potential difference in action between the two anti-TNF agents tested.

25 IL-20 is expressed in inflamed synovium of patients with psoriatic arthritis and rheumatoid arthritis TJM Smeets1, Y Chandrasekher2, JJ Harisingman1, PP Tak1
1Division of Clinical Immunology and Rheumatology, Academic Medical Center/University of Amsterdam, The Netherlands; 2Department of In Vitro Biology, ZytoGenetics, Inc., Seattle, WA, USA Arthritis Res Ther 2004 6(Suppl 1):25 (DOI 10.1186/ar1067)

Background and Objective IL-20 is a novel cytokine that is involved in skin inflammation, in part by serving as an autocrine factor for keratinocytes. The aim of this study was to investigate the expression of IL-20 in the inflamed synovium of patients with psoriatic arthritis (PsA) and rheumatoid arthritis (RA) to provide more insight into the possible role of this cytokine in inflammatory joint disease. To further assess the regulation of IL-20 expression, in vitro experiments were conducted to determine the effects of IL-1β and TNF-α on the expression of IL-20 by RA fibroblast-like synoviocytes (FLS).

Methods Synovial biopsy specimens of patients with PsA (n = 11) and RA (n = 10) were obtained from actively inflamed joints by needle arthroscopy. Immunohistologic analysis was performed using a monoclonal antibody (mAb) specific for IL-20. In addition, we measured IL-20 expression in RA FLS in the presence or absence of 250 pg/ml IL-1β and 100 ng/ml TNF-α by immunocytochemistry.

Results IL-20 was expressed in the intimal lining layer, in the synovial sublining and on endothelium in both PsA and RA patients. Digital microscopic analysis of synovial tissue revealed clear IL-20 expression with comparable overall scores in both patient groups (RA 149775 ± 56466, PsA 123862 ± 50630 [mean integrated optical density ± SEM]; NS). The in vitro experiments revealed marked induction of IL-20 after stimulation with IL-1β and TNF-α in RA FLS.

Conclusion IL-20 expression is induced in FLS by proinflammatory cytokines, including TNF-α and IL-1β. In addition, IL-20 is markedly expressed in psoriatic skin lesions, but also in the synovium of patients with PsA and RA, suggesting a role in various forms of inflammatory joint disease.

26 Cross-talk between TRAIL and TGF-β in regulation of collagen production in scleroderma lung disease VV Yurovsky

University of Maryland School of Medicine, Baltimore, MD, USA Arthritis Res Ther 2004 6(Suppl 1):26 (DOI 10.1186/ar1068)

Background Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a TNF family member, which has been closely associated with...
regulation of the immune response and pathogenesis of autoimmune diseases. The expression of TRAIL was found in CD8 T cells that had undergone oligoclonal expansion in the lungs of patients with systemic sclerosis (scleroderma) and were able to stimulate collagen production in lung fibroblasts in vitro. Among the family members, TRAIL displays highest homology to Fas ligand, the receptor of which may mediate not only cell apoptosis but also the proliferation of normal human fibroblasts.

**Objective** Considering structural and functional similarities between TRAIL and Fas ligand, we examined the effects of soluble multimerized TRAIL on normal human lung fibroblasts.

**Methods and Results** TRAIL at doses in excess of 100 ng/ml induced apoptotic death of fibroblasts. However, at lower concentrations it was found to stimulate collagen production. Collagen alpha2(I) mRNA expression was assessed by real-time RT-PCR, and total soluble collagen was measured in culture supernatants using the Sircol assay. Both alpha2(I) collagen mRNA and total soluble collagen were increased upon TRAIL stimulation, with peak response (more than fourfold increase in mRNA) at 1 ng/ml TRAIL. DNA microarray analysis revealed TRAIL-induced increase in the expression of a number of genes involved in tissue remodeling, including those related to the transforming growth factor (TGF)-β pathway. The microarray results were confirmed by Northern blot analysis of TGF-β1 mRNA expression and by measurements of total active TGF-β1 in culture supernatants. In addition, antibody-mediated blocking of TGF-β1 was shown to abrogate TRAIL-induced collagen synthesis.

**Conclusion** These data suggest that TRAIL can enhance extracellular matrix production in fibroblasts by triggering TGF-β expression, which acts in an autocrine manner. If this process continues uncontrolled, it may contribute to the development of fibrosis, particularly in the lungs of patients with scleroderma.

### 27 Effects of reduced ATP-formation on cytokine production and proliferation in human peripheral CD4+ T cells

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**Background and Objective** The function of immune cells is dependent upon a constant and adequate supply of energy (ATP), which is mainly formed by oxidative phosphorylation (OXPHOS). In arthritis, microenvironmental conditions are characterized by low levels of oxygen and glucose. Thus, effector cells of the innate immune system are recruited to sites where they face an acute need to respond to these demanding conditions. We investigated how immune cells maintain viability and function under these circumstances, and which immune processes are limited to what extent by energy deficiency.

**Methods** From peripheral mononuclear cells obtained from healthy donors, we isolated CD4+ T-cells (MACS, >98% purity) and incubated them (37°C, 5% CO2) in RPMI 1640 with 11.1 mmol/l glucose (permits OXPHOS and glycolysis) and without glucose (permits OXPHOS only). As a measure of oxidative ATP formation, cellular oxygen consumption was determined amperometrically with a Clark electrode. Under the conditions of unaffected ATP production and ATP production inhibited stepwise using myxothiazole, PMA/ionomycin-stimulated cytokine production (IL-2, IL-4, IFN-γ, TNF-α, 6 hours) and anti-CD3/-anti-CD28-stimulated proliferation (over 96 hours) were quantified.

**Results** In the glucose-containing medium, both stimulated cytokine production and proliferation were unaffected, even under complete suppression of OXPHOS. Only when OXPHOS and glycolysis were suppressed simultaneously and almost completely were cytokine production and proliferation significantly decreased.

**Conclusions** We have quantified the energy requirement of specific immune functions in human CD4+ T cells. Under maximally inhibited OXPHOS, glycolysis fully compensates for the ATP supply for the energy requirements of the immune functions investigated. These data demonstrate a high adaptive potential of CD4+ T cells to maintain specific immune functions even under massively impaired energetic conditions.

### 28 Intra-articular steroid treatment reduces synovial HMGB1 but not IL-1 expression in chronic arthritis

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**Objective** In this study we examined the effect of glucocorticosteroids (GCs) in chronic arthritis on IL-1α, IL-1β, TNF and the newly discovered proinflammatory cytokine HMGB1, which was previously thought of only as a DNA-binding protein.

**Methods** Seventeen chronic inflammatory arthritis patients with swelling and pain in at least one large joint were chosen. Synovial biopsies were sampled by arthroscopy before and 9–12 days after intra-articular injection of 40 mg triamcinolone hexacetonide. Synovial membrane T cells (CD3), macrophages (CD68, CD163), as well as the proinflammatory cytokines IL-1α, IL-1β, TNF and HMGB1 were studied by immunohistochemistry followed by semiquantitative analysis and/or digital image analysis.

**Results** All investigated joints had clinical signs of active arthritis with swelling and pain of the joint before treatment, which was reduced in all joints after treatment. Numbers of T cells were reduced whereas numbers of macrophages were not significantly changed. Expression of HMGB1 was significantly reduced, whereas no significant effects were seen on the expression of IL-1α, IL-1β or TNF. Before treatment HMGB1 staining was mainly extracellular and cytoplasmic in the mononuclear inflammatory infiltrates and lining layer, whereas endothelial cells of scattered capillaries, arterioles and venules had a primarily cytoplasmic staining. After treatment staining pattern was more prominent in the nucleus and reduced extracellularly.

**Conclusions** Proinflammatory cytokines in chronic arthritis are affected differently by intra-articular GCs. The marked reduction in HMGB1 expression and the lack of significant change in IL-1 and TNF thus indicate that GCs may exert their in vivo effects in arthritis via partly different pathways than in previous in vitro experiments.

### 29 Human adipose tissue: an important novel source of IL-1Ra

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The increased production of proinflammatory IL-1, TNF-α and IL-6 is part of the pathogenesis of various immunoinflammatory diseases, often accompanied by metabolic and cardiovascular complications. Cachexia and obesity have been shown to accompany these diseases, and the cytokines IL-1, TNF-α, IL-6 are said to be increased in white adipose tissue (WAT). Few studies have been conducted to examine counter-regulatory mechanisms of cytokine inhibitors. Sera of obese patients showed a more than sevenfold increase in IL-1Ra, which matches levels present in inflammatory autoimmune diseases and sepsis, and correlates with body mass index and insulin resistance. Subcutaneous and visceral human WAT of obese patients contained 0.4 and 0.7 mg of IL-1Ra/mg protein, respectively. Thus, in an obese individual weighing 120 kg with 50% body fat, total WAT is estimated to contain 0.6 mg IL-1Ra protein (i.e. 200 times the amount of IL-1Ra in total serum), thus representing one of the main sources of IL-1Ra production. The
increased IL-1Ra expression – not associated with an increase in IL-1β – argues for an anti-inflammatory, compensating mechanism associated with obesity. Our experiments with human WAT explants showed a strong stimulatory effect of PMA and, more importantly, of IFN-β (as much as 5- to 10-fold). Because the latter is a fibroblast-derived IFN, it is tempting to speculate that stromal cells and adipocytes might be part of a paracrine mechanism that regulates IL-1Ra secretion. In fact, newly formed adipose tissue is often found close to inflammatory lesions (i.e. synovial tissue). The functional consequences of the increased production of IL-1Ra by adipose tissue may represent an important counter-regulatory mechanism to inflammation at the local level. Furthermore, IL-1RI and IL-1 IL-1RaCsp were also expressed in human WAT. Thus, substances that increase the production ratio of IL-1Ra/IL-1β by adipose tissue might serve as a novel target for therapeutic strategies in human immunoinflammatory diseases.

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IL-1-dependent cartilage damage in a macrophage-driven arthritis model can be circumvented by T cell IL-17

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Background In the murine macrophage-mediated SCW-induced arthritis, IL-1 plays a dominant role in cartilage destruction, as was shown in previous studies using cytokine gene knockout mice or antibodies against IL-1. T cell IL-17 is a proinflammatory cytokine that has many IL-1-like activities. IL-17 is expressed in the synovium of RA patients and may play a role in rheumatoid arthritis (RA) pathology.

Objective In the present study we examined the potency of T cell IL-17 to bypass the relative IL-1 dependency of cartilage damage during macrophage-driven SCW-induced arthritis.

Methods SCW-arthritis was induced in IL-1 deficient mice and their C57BL/6 controls by intra-articular injection of 25 μg SCW fragments. Sixteen hours before the induction of arthritis, an adenoviral vector expressing murine IL-17 or a control virus was injected into the knee joint using an adenovirus (AdhIL13) and hIL-13 was detected in synovial washouts after 1, 2, 3, and 7 days. ICA was induced in C57Bl/6 by i.a injection of lysozyme-anti-lysozyme complexes 1 day after AdhIL13 injection. Histology and synovium specimens were taken at days 1, 3 and 7 after ICA onset. Joint inflammation and MMP-mediated VDIPEN expression were determined. mRNA levels of MMP-3, MMP-12, and MMP-13 were detected in the synovium by Q-PCR.

Results AdhIL13 injected in naïve knee joints resulted in 1000 pg/ml hIL-13 after 1 day, and increased to 3000 pg/ml detected at days 2, 3 and 7. Surprisingly, IL-13 did not diminish joint inflammation, because the influx of inflammatory cells was similar in the Adel70 and AdhIL13 group. MMP-mediated VDIPEN expression was not found 1 day after ICA onset because of the early time point. At day 3, IL-13 overexpression resulted in a twofold reduction in VDIPEN expression and a three-fold reduction at day 7, compared with the control group. This decrease might be the result of a declined production of MMPs. However, the mRNA level of MMP-3 was comparable between the two groups, whereas MMP-12 and MMP-13 mRNA levels were three times higher in the IL-13 group.

Conclusion IL-13 overexpression during ICA does not alter the inflammatory response. VDIPEN expression was decreased, but this was not reflected by declined MMP production, suggesting that IL-13 interferes at the level of activation of pro-MMPs.

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IL-13 overexpression during immune complex arthritis diminishes MMP-mediated VDIPEN expression

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Background We recently found that overexpression of the Th1 cytokine IFN-γ during immune complex arthritis (ICA) resulted in deterioration of matrix metalloproteinase (MMP)-mediated VDIPEN expression. On the contrary, overexpression of a Th2 cytokine might protect against cartilage destruction.

Objective The aim of the study was to investigate whether local overexpression of the Th2 cytokine IL-13 by gene transfer is able to diminish MMP-mediated VDIPEN expression during ICA and, if so, what mechanism is involved.

Methods Human IL-13 (hIL-13) was locally overexpressed in the knee joint using an adenovirus (Addel70) and hIL-13 was detected in synovial washouts after 1, 2, 3, and 7 days. ICA was induced in C57Bl/6 by i.a injection of lysozyme-anti-lysozyme complexes 1 day after AdhIL13 injection. Histology and synovium specimens were taken at days 1, 3 and 7 after ICA onset. Joint inflammation and MMP-mediated VDIPEN expression were determined. mRNA levels of MMP-3, MMP-12, and MMP-13 were detected in the synovium by Q-PCR.

Results Addel70 injected in naïve knee joints resulted in 1000 pg/ml hIL-13 after 1 day, and increased to 3000 pg/ml detected at days 2, 3 and 7. Surprisingly, IL-13 did not diminish joint inflammation, because the influx of inflammatory cells was similar in the Addel70 and AddhIL13 group. MMP-mediated VDIPEN expression was not found 1 day after ICA onset because of the early time point. At day 3, IL-13 overexpression resulted in a twofold reduction in VDIPEN expression and a three-fold reduction at day 7, compared with the control group. This decrease might be the result of a declined production of MMPs. However, the mRNA level of MMP-3 was comparable between the two groups, whereas MMP-12 and MMP-13 mRNA levels were three times higher in the IL-13 group.

Conclusion IL-13 overexpression during ICA does not alter the inflammatory response. VDIPEN expression was decreased, but this was not reflected by declined MMP production, suggesting that IL-13 interferes at the level of activation of pro-MMPs.

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Overexpression of TNF causes bilateral sacroilitis

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Objective To study the role of TNF in sacroilitis using a TNF-α transgenic (hTNFtg) mouse model.

Figure 1

PG depletion 10 days after induction of SCW arthritis, in combination with the ia injection of control virus or AdhIL17.
Methods hTNFtg mice were divided into two groups receiving either phosphate-buffered saline (PBS) or anti-TNF (infliximab). Wild-type mice served as negative control. Treatment was initiated at week 4 and continued over 6 weeks. Thereafter the sacroiliac joints were histologically assessed for joints inflammation, local bone erosion and cartilage destruction.

Results. All hTNFtg mice showed a severe bilateral sacroiliitis. Treatment of hTNFtg mice with anti-TNF, however, resulted in a significant ($P < 0.05$), over 80% reduction in sacroiliacal inflammation. Furthermore, in hTNFtg mice severe erosions of the iliac as well as sacral subchondral bones were detectable, whereas treatment with anti-TNF virtually abrogated local bone erosions indicated by a reduction by over 99%. In addition, application of anti-TNF also significantly ($P < 0.05$) reduced the numbers of osteoclasts at the front of erosions by 98% compared with PBS-treated hTNFtg mice. The amount of sacroiliac cartilage of hTNFtg mice was significantly ($P < 0.05$) reduced by 78% compared with anti-TNF treated mice.

Conclusion. These data clearly indicate that TNF overexpression causes bilateral, erosive sacroiliitis and that anti-TNF therapy is a suitable tool with which to treat this condition.

Major histocompatibility complex

33 Characterization of the T cell response to hnRNP-A2 (RA33) in HLA-DR4 transgenic mice

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Background. HnRNPA2 (RA33) is a multifunctional RNA-binding protein involved in various aspects of post-transcriptional regulation of gene expression. Autoantibodies to A2/RA33 are present in approximately 30% of rheumatoid arthritis (RA) patients whereas autoreactive T cells have been found in almost 60% of the patients.

Objective. The aim of the study was to analyze the T cell response to A2/RA33 and its possible involvement in the pathogenesis of RA.

Methods. The Tepitope programme as well as ELISAs were used to define peptide sequences within A2/RA33 that may bind to the RA associated MHC molecules HLA-DR*0401 and DR*0404. Candidate peptides were analyzed in vivo by immunizing DR*0401 transgenic (tg) mice and restimulating lymph node cells or purified CD4+ or CD8+ T cells with the peptides. The potential of A2/RA33 to induce disease was studied by immunizing arthritis-prone DBA/1 mice, a strain bearing the DR*0401 transgene on the DBA/1 background and TNF tg mice (which spontaneously develop erosive arthritis).

Results. Immunogenic sequences were found clustered in the RNA binding domains of A2/RA33. In immunized HLA-DR4 tg mice the reaction to A2/RA33 peptides seemed to be entirely produced by CD4+ T cells showing a Th1 phenotype, with some of the peptides inducing high production of IFN-γ. However, immunization of HLA-DR4 tg or DBA/1 mice with A2/RA33 protein induced only mild swellings in some animals with no pathological findings in the histological analysis. On the other hand, immunization of TNF tg mice enhanced arthritis significantly. These mice overexpressed A2/RA33 in the joint, whereas expression was very low in wild-type mice.

Conclusion. A2/RA33 derived peptides can bind to RA associated HLA class II molecules and induce a proinflammatory Th1 response. The increased arthritis observed in immunized TNF tg mice suggests that the autoimmune response to A2/RA33 may indeed be involved in the pathogenesis of RA by inducing or enhancing disease in a proinflammatory environment.

34 Association between HLA class II genes and autoantibodies to cyclic citrullinated peptides (CCP) affects severity of rheumatoid arthritis

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Objective. The role of HLA class II molecules in the pathogenesis of rheumatoid arthritis (RA) is unclear. HLA class II molecules are involved in the interaction between T and B lymphocytes required for long-lived B-cell responses and generation of high-affinity IgG antibodies. The relationship between HLA class II polymorphisms and RA-specific IgG anti-cyclic citrullinated peptide (CCP) antibodies was investigated.

Methods. High-resolution HLA-DR and DQ typing and anti-CCP2 antibody testing was performed in 279 RA patients from the Leiden Early Arthritis cohort. The presence of anti-CCP antibodies was analyzed in carriers of the different DR and DQ alleles. Disease progression was measured over a period of 4 years by scoring radiographs of hands and feet using the Sharp/Van der Heijde method.

Results. Carriers of DQ-DR genotypes containing proposed RA susceptibility alleles were significantly more frequently anti-CCP antibody positive. Carriership of one or two HLA-DRB1 shared epitope (SE) alleles was significantly associated with production of anti-CCP antibodies (OR 2.9, 95% CI 1.6–5.2 and OR 10.2, 95% CI 3.8–28.5, respectively). In SE carrying, anti-CCP antibody positive patients an increased rate of joint destruction was observed (mean Sharp score 7.6 points/year) compared with non-SE carrying, anti-CCP positive (2.4 points; $P = 0.03$), SE carrying, anti-CCP-negative patients (1.6 points; $P < 0.001$) and non-SE carrying, anti-CCP negative patients (1.6 points; $P < 0.001$).

Conclusions. Anti-CCP antibodies are associated with HLA class II RA susceptibility alleles and presence of both factors is indicative of a severe disease course. As the principal role of HLA class II molecules is antigen presentation to T cells, these data point toward a pivotal role of citrulline-directed T cells in the production of anti-CCP antibodies.

35 HLA-DRB1 encoded predisposition to rheumatoid arthritis: the anchor hypothesis

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Objective and Methods. Antigenic peptides anchor by amino acid residues to pockets within the antigen-binding groove of the HLA-DRB1 molecule. In order to assess whether a pocket-wise analysis of HLA-encoded susceptibility might provide a better fit to the immunogenetic data in rheumatoid arthritis (RA) than the shared epitope hypothesis, HLA-DRB1 typing findings in 167 patients with recent onset RA and in 166 controls were analyzed.

Results. The shared epitope (residues 67–74) borders pockets 4 and 7. After stratification for pocket 4, polymorphisms in pocket 7 did not influence RA risk. However, after stratification for pocket 7, both substitutions in the α helix bordering pocket 4 and substitutions at position 13 in the floor of this pocket independently and significantly influenced RA risk. Furthermore, substitutions at position 86 in pocket 1 also had an effect on RA risk. Pairs of HLA-DRB1 alleles that only differ in the glycine/valine dimorphism showed a consistent tendency toward increased risk for the glycine variant in a pooled analysis (pooled OR...
1.66; p1 = 0.08). This observation is also strongly supported by data from previous publications.

Conclusion Structural data indicate that HLA disease association may be determined by the make-up of individual pockets within the antigen-binding groove of the HLA molecule. In RA, susceptibility is influenced by genetic polymorphisms in pocket 4 of the HLA-DRB1 molecule, both in the floor of this pocket and in the α helix bordering this pocket. In addition, genetic variation in pocket 1 modifies RA risk.

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Influence of the physicochemical properties of the HLA-DRB1 shared epitope negative alleles on rheumatoid arthritis susceptibility

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Objective HLA-DRB1 alleles encoding the shared epitope (SE) are associated with rheumatoid arthritis (RA) but the underlying biological mechanism is unknown. Several case–control studies have been published on the protective effect of particular SE negative alleles, according to the physicochemical properties of the fourth antigenic peptide binding pocket (P4). The present study was undertaken to test those hypotheses taking advantage of a familial based association analysis.

Methods One hundred French Caucasian families with one RA patient and both parents were genotyped for HLA-DRB1. The analysis relied on the allelic frequency calculation, the transmission disequilibrium test (TDT) and the genotype relative risk analysis (GRR).

Results After the demonstration of the association of SE with RA in our sample (P = 4.8 × 10^{-14}), we replicated the strong protective effect of HLA-DRB1*1501 alleles (P = 4.77 × 10^{-7}). We also replicated a protective effect against RA of the alleles with an isoleucine at position 67 (P = 1.93 × 10^{-5}) and the alleles with an aspartic acid at position 70 (P = 3 × 10^{-3}). We failed to replicate any protective effect of the alleles with a neutral or negative electric charge of the P4 pocket, nor a modulation of SE effect by those alleles. We also failed to replicate the protective effect of Q and De alleles according to the functional categorization of HLA-DRB1 alleles.

Conclusion Our findings are in keeping with a protective effect against RA of SE negative alleles (HLA-DRB1*1501, alleles with an isoleucine at position 67 and alleles with an aspartic acid at position 70).

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Autoreactive T cells specific for a natural intracellular hapten: an example from patients with acute myocarditis and possible consequences for other inflammatory diseases

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Background All organisms contain naturally occurring haptens in the form of enzyme cofactors bound covalently to apoproteins. One example is flavine adenine dinucleotide (FAD). Autoantibodies recognizing flavoproteins, with specificity for flavin or flavin-peptide were described in patients with myocarditis or other muscular diseases.

Objective We wanted to test the hypothesis that FAD-peptide reactive T cells exist in patients with acute myocarditis.

Methods Peripheral blood mononuclear cells (PBMCN) were tested in vitro with a purified FAD-peptide (from a trypsin digested, affinity purified flavoprotein) or a synthetic peptide with the same amino acid sequence. Proliferation was measured by 3H-TdR incorporation, and the secretion of IFN-γ by ELISA.

Results PBMCN from four patients with acute myocarditis showed positive responses to the FAD-peptide, in contrast to control individuals. The synthetic FAD-free peptide did not induce a response.

Conclusions The results are consistent with the hypothesis that during the inflammation of the heart cardiomyocytes liberate normally cryptic mitochondrial FAD-peptides, which induce a T cell response. Similar mechanisms could be envisaged in inflammatory diseases of other compartments (e.g. the joints in rheumatic diseases [1]).

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Reference


T-cells

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CD28nullCD4+ T cells: an effector memory T cell population in patients with rheumatoid arthritis

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Background CD4+ T cells lacking the costimulatory molecule CD28 have been described in both elderly individuals and in several chronic inflammatory disorders, one being rheumatoid arthritis (RA).

Objective We characterized such CD28nullCD4+ T cells from RA patients in order to identify surface markers correlating with function.

Methods Peripheral blood mononuclear cells were analyzed for surface marker expression by flow cytometry and in vitro cultures were set up for functional studies.

Results One-third of the RA patients had a persistent and expanded CD28null population, which comprised up to half of the CD4+ T cells in peripheral blood. Functionally, CD28nullCD4+ T cells were potent effector memory cells with regard to their proliferation and cytokine secretion profiles. This functional capacity correlated with a hitherto unpublished surface phenotype, the cells being uniformly CCR7-negative and CD43high. In addition, we re-evaluated previously suggested cell surface markers, and found CD57 and CD11b expressed on the majority of these NKT-like CD4+ T cells. When combining phenotypic and functional analyses of subpopulations of the CD28nullCD4+ T cells a new terminally differentiated T cell population was identified.

Conclusion We believe that in the balanced immune system of healthy individuals, CD28nullCD4+ T cells are under homeostatic control, whereas in an unbalanced immune system such as in autoimmune disease CD28nullCD4+ T cells are allowed to expand and have the capacity to enhance ongoing inflammatory reactions.
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T cell response in Shigella-induced reactive arthritis
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Background Shigella flexneri is one of the bacteria causing reactive arthritis (ReA). However, no target antigen for the cellular and humoral immune response against Shigella is yet known.

Methods Shigella were grown in culture followed by differential centrifugation, after which a cytoplasmic and the 70S ribosomal fraction were obtained. By means of density gradient centrifugation the 70S ribosome was divided into its two subunits, the small ribosomal subunit 30S and the large ribosomal subunit 50S. The proteins derived from both subunits were further separated by FPLC–ion exchange chromatography. Mononuclear cells from synovial fluid of 3 HLA-B27 positive patients with Shigella-induced ReA were stimulated with whole Shigella, the cytoplasmic fraction, the ribosomal subunits and ten single protein fractions from the ribosomal subunits gained by FPLC. Stimulatory protein fractions were subsequently cleaned by RP-HPLC and identified by stamp-size 2D PAGE.

Results Seventeen protein fractions from the 30S subunit and 23 protein fractions from the 50S subunit were obtained. A lymphocyte proliferation was seen after stimulation with whole Shigella, with the cytoplasmatic fraction and the two ribosomal subunits but not with control antigens. More interestingly, five single ribosomal proteins out of 40 were equally stimulatory in all three patients, indicating that they represent immunodominant T cell antigens. Characterization by 2D PAGE identified the ribosomal proteins L15, L18, L21, L22 and L23 from 50S (L’ for large subunit) and S3 from the 30S (S’ for small subunit).

Conclusion This approach allows the identification of T-cell epitopes derived from bacteria in ReA patients. In a first step we identified proteins from the highly conserved ribosomal fraction of Shigella flexneri. Their role in the pathogenesis must be further investigated.

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CD25+ regulatory T cells can be used therapeutically in collagen-induced arthritis
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Background Naturally occurring CD4+CD25+ T cells have been shown to suppress immune responses both in vivo and in vitro. Finding a way to harness their regulatory abilities is of particular value in both transplantation and autoimmunity, where unwanted immune reactions need to be eliminated. Tantalizing evidence for their future potential is demonstrated by their ability to cure T cell mediated colitis induced in SCID mice [1]. However, many autoimmune diseases display a strong humoral component, such as rheumatoid arthritis. It is unknown whether CD4+CD25+ T cells are effective in these B cell-mediated autoimmune diseases.

Objective We tested the ability of CD4+CD25+ T cells to modulate collagen-induced arthritis (CIA), a disease dependent on the presence of B cells [2].

Methods In order to determine the role of endogenous CD25+ T cells in CIA, CD25+ cells were first depleted in mice before immunizations to induce CIA. The therapeutic value of these cells was assessed by adoptively transferring preactivated CD4+CD25+ T cells into mice during the onset of disease.

Results Depletion of CD25+ cells before CIA induction lead to a hastened severe disease in comparison with nondepleted control mice. If CD4+CD25+ T cells were transferred to mice therapeutically, the mice had a significantly milder disease.

Conclusion CD4+CD25+ T cells can treat chronic arthritis and probably have the potential to treat a wide spectrum of autoimmune diseases, including those that are mainly mediated by B cells.

References

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Characterization of kidney infiltrating T cells in the NZB/W F1 lupus model
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Background Lupus nephritis is one of the hallmarks of systemic lupus erythematosus (SLE). Although there is increasing evidence that these T lymphocytes might play a major role in the initiation and maintenance of nephritis, there is hardly any functional data about these cells.

Objective The aim of the present study was to characterize kidney infiltrating T cells with respect to their activation state, their memory/effector phenotype, proliferation, and cytokine production. Furthermore, the ability to provide T cell help for the generation of autoantibodies was tested.

Methods CD4+ T cells derived from inflamed kidney tissues of 7- to 9-month-old NZB/W F1 mice were analyzed for the expression of CD69, CD25, CD28, CD62L, CD45RB, CD44, and CD71 by FACS. Furthermore, intracellular cytokine staining was measured after PMA/Ionomycin stimulation, and the ability of kidney T cells to respond to the SmD1 83-119 peptide with increased generation of anti-dsDNA antibodies was tested by ELISPOT.

Results and Conclusion The kidney infiltrating CD4+ T cells were highly activated in terms of CD69 expression (28%). The expression of CD25 was lower compared with splenic T cells (8.6% versus 15.2%). The CD4+ T cells were mainly of the effector/memory phenotype (55–60%), but there was also a high frequency of naïve T cells (40–45%). There was a low frequency of proliferating T cells (5%), as detected by CD71 expression. Most T cells were of the Th1 phenotype and produce proinflammatory cytokines such as IFN-γ (16.8%) and TNF-α (11.8%). Kidney T cells give no help for the generation of anti-dsDNA antibodies.

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CD25brightCD4+ regulatory T cells accumulate in inflamed joints of patients with chronic rheumatic disease
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Background CD25+CD4+ regulatory T cells participate in the regulation of immune responses. We recently demonstrated an enrichment of CD25brightCD4+ T cells with a capacity to control T cell proliferation in the joints of patients with rheumatoid arthritis (RA).

Objective Here, we extend these studies to investigate a possible accumulation of regulatory T cells in the joints of other inflammatory diseases, and their potential to suppress cytokine production in patients with rheumatic diseases, including RA.
Methods Synovial fluid and peripheral blood samples were obtained during relapse from 204 patients with spondyloarthropathies, juvenile chronic arthritis, and RA, and the frequency of CD25brightCD4+ T cells was determined. For functional studies, synovial cells from six patients were sorted by flow cytometry and the suppressive capacity of these CD25brightCD4+ T cells was determined in vitro coculture.

Results Of 204 patients, 198 patients exhibited a higher frequency of CD25brightCD4+ T cells in synovial fluid as compared with peripheral blood. Additionally, in comparison with healthy individuals, the patients had significantly fewer CD25brightCD4+ T cells in peripheral blood. Functionally, the CD25brightCD4+ T cells suppressed both type 1 and 2 cytokine production, and proliferation, independent of diagnosis.

Conclusion Irrespective of diagnosis of the inflammatory joint disease, an accumulation of CD25brightCD4+ T cells in the joint and reduced numbers in peripheral blood suggest an active recruitment of regulatory T cells to the affected joint. Their capacity to suppress both proliferation and cytokine secretion might contribute to dampen the local inflammatory processes.

Dendritic cells

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Increased expression of chemokines DC-CK1, ELC and TARC by dendritic cells and in synovium from patients with rheumatoid arthritis

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Background Dendritic cells (DCs) are the professional antigen-presenting cells that produce a large set of chemokines. Recent evidence suggests that DCs, by their production of chemokines, are important in the inflammatory cascade.

Objective The aim of the study was to evaluate the expression of chemokines by DCs and synovial tissue from patients with RA in comparison with that from healthy individuals.

Methods Immature and mature DCs were obtained using standardized protocols as described previously. The expression of the chemokines DC-CK1, ELC, IL-8, MIP-1α, SDF-1α, SDF-1α, lymphotactin, SLC, MIP-3α, TARC and MDC in iDCs, mDCs and synovial tissue was determined by using real-time quantitative RT-PCR techniques (PRISM).

Results iDCs of RA patients (n = 10) express low levels of ELC and MIP-1α, and high levels of MDC and TARC, which are equal when compared with DCs from healthy individuals (n = 8). However, the expression of DC-CK1 (P < 0.001) and IL-8 (P = 0.02) by iDCs from RA patients was significantly higher. After full maturation, expressions of DC-CK1 (P < 0.001), ELC (P < 0.001), IL-8 (P = 0.02), MIP-1α (P < 0.001) and TARC (P < 0.001) were significantly higher in DCs from RA patients relative to healthy donors. In RA synovial tissue, DC-CK1 (230-fold), ELC (400-fold) and IL-8 (36-fold) were expressed at much higher levels when compared with healthy individuals. TARC was also expressed at higher levels in RA synovial tissue but this failed to achieve significance.

Conclusion In DCs both from patients with RA and in biopsies of rheumatoid synovium, significantly higher levels of DC-CK1, ELC and IL-8 mRNA are present relative to their normal counterparts. MIP-1α and TARC were expressed at higher levels exclusively by DCs from RA. These results suggest that DC-CK1, ELC, MIP-1α and TARC, along with DCs, play a critical role in RA.

Effect of an empty plasmid containing CpG on dendritic cells: in vitro steady state of stimulation, in vivo prevention of collagen-induced arthritis

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Objective We studied the immune response against pcDNA3.1 in a model of rheumatoid arthritis (RA), namely collagen-induced arthritis (CIA), to understand the role of innate immunity in RA and the incapacity of this empty plasmid used in gene therapy.

Methods CIA was induced in DBA/1 mice by type II collagen sc injections. At D10 and D24 after immunization, 1 μg pcDNA3.1neo (CpG islets-containing plasmid) or pCor (control plasmid) were injected in lower limbs. DBA/1 BMDC were stimulated in vitro with pcDNA3.1neo, pCor, LPS +/− TNF for 24 and 48 hours. BMDC were analyzed in flow cytometry, and IL-12, TNF-α, IFN-γ and IL-10 were tested by ELISA in BMDC supernatants. BMDC endocytosis capacity and MLR functional capacity were also studied. CIA was treated with a stimulated dendritic cell population after immunization.

Results PcDNA3 decreases the incidence of arthritis in collagen immunized DBA mice. Stimulation of BMDC with pcDNA3 increased costimulation molecule expression, cytokine production (IL-12, TNF-α, IFN-γ and IL-10), and allogeneic T cell proliferation, but at a lower level than LPS stimulation. Moreover, pc DNA3 stimulated BMDC have higher endocytosis capacity than LPS stimulated BMDC. Treatment of collagen immunized mice with this steady-state activated-dendritic cell population decreases the incidence of arthritis.

Conclusion We demonstrated that the stimulation of dendritic cells with an empty plasmid, pcDNA3.1neo, could lead to a steady state of activation that could support tolerance induction. This effect needs to be further characterized and seems to be a ‘yes’ or ‘no’ answer, as incidence is decreased but total clinical scores are not statistically modified. Histological studies will help to understand this mechanism.

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RANK and RANKL expression in rheumatoid arthritis synovium and lymph nodes as markers of dendritic cell–T cell interactions

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Background The RANK/RANKL pathway is critical in osteoclastogenesis and bone destruction, and is implicated in focal bone erosion in rheumatoid arthritis (RA). Because deficient mice show major lymph node (LN) abnormalities, here we looked at its involvement in the immune response during chronic inflammation.

Methods We investigated, by immunohistochemistry, RANK and RANKL expression by DC and T cell subsets in paired RA synovium–LN and also in normal peripheral blood mononuclear cells (PBMC) stimulated with PMA/PHA.

Results In RA synovium, RANKL+ cells were detected in the lining layer and the lymphocytic infiltrates whereas RANK expression was restricted to the perivascular infiltrates. In LN, RANK+ and RANKL+ cells were diffusely expressed in both T-cell zone and germinal centres. Double staining with anti-RANK or anti-RANKL and anti-CD1a or anti-DC-LAMP antibodies revealed that, in paired RA synovium-LN sections, some immature CD1a+ DC express RANK and RANKL whereas some mature DC-LAMP+ DC expressed only RANK. Double staining with the CD3, CD4 T-cell markers and the IFN-γ and IL-17 Th1 cell markers showed that some of CD3+, CD4+, IFN-γ+ and IL-17+ cells expressed RANKL, whereas none of them expressed RANK. The same
Differential dendritic cell expression in chronic inflammatory arthritis: implications for their maturation, recruitment and involvement in disease
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Background Dendritic cells (DC) from both myeloid and lymphoid haematopoietic lineages comprise a complex network of professional antigen-presenting cells, directly linking innate and adaptive immunity. Although implicated in the pathogenesis of inflammatory arthritides, their subset analysis has been hampered by a lack of specific DC markers and reliable quantitation.

Objectives The study was conducted to quantify circulating plasmacytoid DC (pDC) and myeloid DC (mDC) populations in different chronic inflammatory arthritides utilizing a novel assay involving the DC-specific markers BDCA-1 and BDCA-2.

Methods Peripheral blood (PB) was obtained from RA (n = 12), PsA (n = 13), AS (n = 11) and healthy donors (n = 12). Synovial fluids (SF) were also examined from a subset of PB samples (4 RA, 2 PsA, 1 AS).

Results Circulating PB mDC populations were significantly decreased in RA patients (P = 0.004), but not in PsA or AS patients, compared with healthy donors (normal = 175 × 10², RA = 95.5 × 10², PsA = 141 × 10², AS = 152 × 10²). Circulating PB pDC populations were significantly decreased in RA (P = 0.01) and PsA (P = 0.01) but not AS patients (normal = 137 × 10², RA = 54.6 × 10², PsA = 69.5 × 10², AS = 104 × 10²). The mDC:pDC ratio in PB of all patient groups was similar to that in healthy donors, whereas mDC exceeded pDC in SF in all patient cohorts analyzed (RA 6.6:1, PsA 7:3:5:1 and AS 100:1).

Conclusions We report the ex vivo tracking of circulating pDC and mDC populations, revealing significantly reduced numbers of pDC in RA and PsA and mDC in RA patients. mDC subsets appear to be the predominant population in SF in all patient cohorts studied. Understanding the biology of DC subsets in chronic inflammatory arthritis will elucidate their presumed critical role at the interface of innate and acquired (autoimmune) immune responses.

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Triggering of FcγR during dendritic cell maturation leads to a decreased expression of the chemokines DC-CK1, ELC and TARC
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Introduction Dendritic cells (DCs) determine the balance between tolerance and immunity in which Fcγ receptors (FcγR) are thought to play a decisive role. Previously, we found that DCs from RA patients expressed a different FcγR profile.

Objective The aim of the study was to determine the potential difference in chemokine expression and production between DC from RA patients and healthy controls upon FcγR mediated triggering.

Methods Immature and mature DC were obtained using standardized protocols as described recently. The expressions of the chemokines DC-CK1, ELC, IL-8, MIP-1α, TARC and MDC were determined by using real-time quantitative RT-PCR techniques (PRISM). The production of DC-CK1 was confirmed by using ELISA techniques. Triggering of FcγR was achieved by the addition of heat aggregated IgG immunoglobulins (HAGGS) to the culture for 48 hours.

Results Stimulation of FcγR resulted in a significantly decreased expression of DC-CK1 (39%), ELC (50%), IL-8 (640%) and TARC (39%) by DC from RA patients (n = 6). Although not significant, the same trend was observed for MIP-1α (160%). In contrast, a significant increase was seen for DC-CK1 (300%), ELC (120%), IL-8 (640%), MIP-1α (160%) and TARC (39%) upon triggering of FcγR on DC from healthy individuals (n = 5). MDC was the only chemokine that showed a decrease upon FcγR dependent stimulation of DC in both groups. By using ELISA techniques, we confirmed these data for DC-CK1 on the protein level. Triggering of FcγR led to a decreased (63%) production of DC-CK1 by DC from RA patients, whereas an increase (157%) was observed by those from controls.

Conclusion Our study lends strong support for a skewed FcγR balance toward the inhibitory subtype in RA, and shows the critical role for FcγR in the inhibition of chemokine production by DCs. Furthermore, our data show that FcγR triggering influences Toll-like receptor (LPS) mediated cell responses.
Phenotypic and functional deficiencies of monocyte derived dendritic cells in systemic lupus erythematosus patients

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Background Systemic lupus erythematosus (SLE) represents an autoimmune disease for which alterations in T cells, B cells as well as various antigen-presenting cell (APC) populations have been described.

Objective In order to better define APC-associated deficiencies we analyzed morphologic, phenotypic and functional characteristics of dendritic cells (DC) from SLE in patients as compared with healthy controls.

Methods Monocyte derived DC (MoDC) were generated by culturing monocytes with GM-CSF-HL-4 for 8 days and with LPS for additional 2 days. MoDC were analyzed at different stages of maturation for morphologic, phenotypic and functional characteristics.

Results and Conclusion Analysis of MoDC at different stages of maturation revealed substantial phenotypic and functional defects of MoDC from SLE as compared with healthy controls. In particular, we observed a significantly reduced upregulation of MHC class II molecules on MoDC upon activation, which correlated with disease activity scores and functional deficiencies in mixed lymphocyte reaction experiments. Our data imply a crucial role of APC in the immunologic imbalance in SLE for foreign- and self-antigen reactivity.

Vaccination with TNF-α modulated DCs protects against CIA in an antigen-specific manner

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Background and Objective Dendritic Cells (DCs) play an important role in initiation and regulation of immune responses in arthritis, because these cells are crucial for the initiation of T cell immunity. Full mobilization of effector T cells depends on proper maturation of DCs. Current evidence indicates that the type of T cell response indeed crucially depends on the activation status of DC. For example, antigen recognition on resting DCs can result in immunoregulation. In this study we explored the immunological effects of differentially matured DCs on the development of collagen-induced arthritis (CIA).

Methods Bone marrow derived DCs were cultured in the presence of GM-CSF with or without IL-4. Immature, TNF-α or LPS activated, antigen-pulsed DCs were used. At several time points before immunization with bovine CII protein, mice were injected with DCs exposed to these different maturation stimuli. Mice were boosted on day 21 post-CII-immunization and disease course was monitored.

Results Although vaccination with immature or LPS activated DCs had no significant influence on disease course, administration of antigen-loaded, TNF-α modulated DCs resulted in a delayed onset of arthritis and a decreased clinical score. The effect was antigen-specific, as also evidenced when antibody titres were measured. A specific increment in the collagen-specific ‘Th1-associated’ IgG3 response was observed. Remarkably, IgG1 was unaffected by this vaccination.

Conclusions CIA can be prevented through vaccination with TNF-α matured DCs in an antigen-specific manner. These findings provide a rationale to employ DCs in treatment or prevention of arthritis.

The effects of NF-κB inhibition by a NEMO binding domain peptide in vitro and in vivo

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Objective Dendritic cells (DC) are thought to play an important role in the pathogenesis of rheumatoid arthritis (RA). In RA, fully matured APC are present in the inflamed synovial tissue clustered around activated T cells. Because DC maturation and cytokine production are NF-κB dependent, we hypothesized that blocking NF-κB activity in DC by selectively targeting IKKβ with a specific NEMO binding domain (NBD) peptide could inhibit the maturation of DC and thereby modulate the immune response.

Methods Immature DC were obtained by culturing freshly isolated monocytes in the presence of GM-CSF and IL-4. On day 6 immature DC were incubated with the NBD peptide (WT versus MUT) in the presence of maturation factors. DC were analyzed for NF-κB activity, surface marker expression, cytokine production and interaction with naive T cells. Furthermore, we investigated the effect of ia injection of the NBD peptide in adjuvant arthritis in rats.

Results NF-κB blockade by the NBD peptide resulted in strongly reduced maturation of DC. In addition, IL-6, IL-12 and TNF-α production was dose dependently blocked. Interestingly, NBD peptide treatment resulted in increased CCR7 expression. Coculture with naive T cells resulted in reduced proliferation and differentiation. In vivo, injection of the NBD peptide resulted in reduced paw swelling in arthritic rats.

Conclusions Inhibiting NF-κB with the NBD peptide in DC results in an immature phenotype with increased CCR7 expression. The enhanced CCR7 expression may promote migration to draining lymph nodes and presentation of antigen in the absence of costimulation. These data strongly suggest that targeting NF-κB in DC could be beneficial in the treatment of arthritis, which is supported by preliminary data in rat adjuvant arthritis.

CD44 regulates bone erosion and osteoclastogenesis in arthritis

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Background and Objective CD44 mediates cell-matrix interaction and is thought to play a role in cell adhesion, fusion and migration. Blocking of CD44 is considered as potential target in the therapy of rheumatoid arthritis.

Methods To elucidate the role of CD44 in arthritis, human TNF transgenic (hTNFtg) mice were crossed with CD44 knockout mice.
Clinical evaluation revealed a significantly increased severity of arthritis in CD44–/– mice compared to hTNFtg mice. Histologically, bone destruction was found to be more severe in hTNFtg mice than in hTNFtg controls. In addition, bone resorption assay showed a significant increase in bone resorption in CD44–/– mice treated with hTNFtg arrays. These results demonstrate that DNA arrays may be used for the detection of specific inhibitors and Fc receptor type II are upregulated by OPG. These results suggest that OPG may be an effective therapeutic target for arthritis.

56 Bone morphogenic protein (BMP-2) enhances expression of type II collagen and aggrecan in human chondrocytes in 3D alginate cultures

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Background The healing capacity of articular cartilage is very limited in the adult. Therefore, tissue engineering techniques were developed to treat cartilage lesions.

Objective Because high expression of type II collagen is of importance for the properties of cartilage after transplantation, we investigated whether the growth factor BMP-2 may modulate the chondrogenic phenotype in monolayer cell cultures (2D) and in three-dimensional culture (3D) systems.

Methods Chondrocytes from articular knee cartilage of five individuals were isolated and expanded under GMP conditions suitable for clinical purposes. Cells were seeded either in 2D cultures or embedded in alginate beads (3D) in the presence or absence of human recombinant BMP-2 (hr-BMP-2). Then, cells were harvested and analysis of the chondrogenic phenotype was performed using quantitative RT-PCR, immunocytochemistry and ELISA methods.

Results Expansion of chondrocytes in primary culture (P0) or in first subculture (P1) did not yield populations enriched for dedifferentiated or hypertrophic cells, because type X collagen encoding mRNA was detectable only at very low copy numbers. Seeding P1 chondrocytes in 3D culture significantly reduced type I collagen, BMP-4 and IL-18, and induced type II collagen and BMP-2 encoding mRNA. Suppression of IL-1β and induction of IL-10 were noted but were not statistically significant. At P2, these changes were still evident. Addition of BMP-2 to 2D chondrocytes had no effect on type II collagen or IL-1β mRNA amounts. In contrast, in 3D chondrocytes BMP-2 induced type II collagen and reduced IL-1 mRNA amounts. This was also seen by ELISA and immunocytochemistry.

Conclusions We conclude that chondrocytes during expansion for ACT may benefit from BMP-2 activation only when seeded in an appropriate 3D culture system.

57 Human bone marrow-derived mesenchymal stem cells: studies on chondrogenic differentiation, chemotaxis and recruitment

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Background We tested the hypothesis that a tissue engineering approach using chemoattractant molecules will allow in situ recruitment of human bone marrow mesenchymal stem cells (MSC) to sites of degenerated articular cartilage.

Objective The aim of our ongoing study is to characterize molecules that are involved in chondrogenesis and chemotaxis of MSC.

Methods Human bone marrow aspirates were used to isolate MSC. The culture homogeneity was verified by FACS analysis (CD14+, CD34+, CD45-, CD105+, CD166+). For differentiation, MSC were centrifuged to form high-density cultures and different TGFs were added. Chondrogenesis was documented by Alcian blue, type II collagen staining and by real-time PCR of marker genes. Chemotaxis was analyzed by chemokine receptor analyses and transwell chamber assays.

Results The histological staining showed BMP-2 promotes differentiation along the chondrogenic lineage. Matrix formation was comparable to MSC stimulated with TGF-β3. The results suggest a synergistic stimulation on chondrogenic differentiation with BMP-2 and TGF-β3. Differentiation was further demonstrated by the induction of type II collagen, aggrecan, SOX-9 and link protein on the gene expression level. Furthermore, for the first time, expression of chemokine receptors of all four chemokine subfamilies was proven by RT-PCR and subsequent sequence analysis, as well as at the protein level using specific antibodies. The chemotactic activity in 24-transwell chemotaxis chambers was demonstrated for, for example, SDF1α.

Conclusion These findings demonstrate that chondrogenic development of MSC is inducible by different TGF-β3 superfamily growth factors including BMP-2. Additionally, MSC express chemokine receptors relevant for chemotaxis or in situ recruitment for cartilage regeneration.

58 Requirement of local synovial IL-17 receptor signaling in the progression of chronic synovitis and bone erosion in arthritis

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IL-17 is a proinflammatory cytokine that is suspected to be involved in development of rheumatoid arthritis (RA). However, the role of IL-17→IL-17 receptor signaling in the effector phase of arthritis, including requirement of this signaling pathway in synoviocytes, is still not fully elucidated. Here we demonstrate, using IL-17 receptor deficient (IL-17R−/−) mice, a requirement for IL-17R signaling in immune reactivity and progression of synovitis and bone erosion in a T cell mediated mBSA arthritis model and chronic streptococcal cell wall arthritis. Of great interest, chimeric mice of host wild-type (wt) and donor IL-17R−/− bone marrow (BM) cells developed chronic synovitis similar to that in wt/wt chimeras. In contrast, chimeric mice of host IL-17R−/−and donor wt BM cells were protected from progressive synovitis similar to IL-17R−/−→IL-17R−/− chimeras, suggesting a crucial role of resident synovial cells. Weakened mobilization of neutrophils into the joint and downregulation of synovial mRNA expression for leukocyte specific chemokines and selectins, and MMP-1 was found in IL-17R−/− mice. These data suggest that, in addition to the role played by IL-17→IL-17R signaling in development of immunity, synovial IL-17→IL-17R signaling plays a critical role in the effector phase of arthritis. Activation of IL-17R on fibroblast-like synoviocytes appears pivotal.

59 TRAIL-induced rheumatoid arthritis fibroblast-like synoviocyte proliferation is inhibited by OPG

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TNF-α related apoptosis inducing ligand (TRAIL) is a proapoptotic factor that can also induce cell proliferation. The role of TRAIL in rheumatoid arthritis (RA) is still unclear. We investigated the effect of TRAIL on RA fibroblast-like synoviocyte (FLS) proliferation. TRAIL induces RA FLS proliferation in a dose-dependent manner, with a maximum proliferation at 0.5 nmol/l (P<0.05; n=5). This proliferation could be prevented by the natural TRAIL inhibitor osteoprotegerin (OPG) and a TRAIL antibody. By flow cytometry, we analyzed TRAIL receptors (DR4, DR5 and DcR2). RA FLS constitutively expressed DR5 (n=5) and three out five RA FLS expressed DR4. Interestingly, RA FLS proliferate more after TRAIL stimulation when expressing both

Available online http://arthritis-research.com/supplements/6/S1
DR4 and DR5, suggesting a cumulative effect of the two receptors. DR5 receptor mediates the signal-inducing cell proliferation because stimulation with an agonistic anti-DR5 antibody induces RA synoviocyte proliferation (P = 4; P < 0.05). Next, we investigated which cells in the synovium could produce TRAIL and OPG. In RA FLS, TRAIL was detected at the mRNA level after IL-1β and TNF-α stimulation but not at the protein level. OPG was constitutively produced (2 ng/ml) and upregulated by IL-1β (14-fold) and TNF-α (5-fold) but not IL-1β. On RA synovial T cells, TRAIL was constitutively expressed. Our results show that TRAIL induces RA FLS proliferation. TRAIL produced on synovial T cells may interact with RA FLS expressing DR4 and DR5 to induce FLS proliferation. OPG inhibits TRAIL induced RA FLS proliferation. This novel property of OPG may be another explanation for its bone protective role in RA.

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Expression of TRAIL-R2 (DR5) on fibroblasts from synovial fluid of rheumatoid arthritis
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Fibroblasts can be isolated from rheumatoid arthritis (RA) synovial fluid (Sfld), and these cells may be derived from the synovium. Although normal tissues do not express DR5 protein, most cancerous tissues express high levels of DR5. Synovial fibroblasts from RA synovium share expression of DR5 protein with transformed cells. Our objective was to characterize fibroblasts from RA Sfld using an anti-DR5 monoclonal antibody. Sfld mononuclear cells were cultured from 50 patients with RA, 20 patients with seronegative arthritis, 20 patients with osteoarthritis (OA), and 15 patients with unclassified monoarthritis or oligoarthritis. Progressive growth of spindle-shaped cells was observed in some cultures. By the third passage 100% of cells were fibroblasts (Thy-1-, CD45+, CD68+). Fibroblasts were stained with a PE-conjugated anti-DR5 antibody and examined by flow cytometry. Fibroblasts grew from 35 of 50 RA Sfld samples. Twenty-six of these 35 were DR5+ (>95% positive cells; MFI relative to isotype control, 13.97 ± 2.5). Fibroblasts also grew from Sfld in six out of 20 seronegative arthritis, four out of 20 OA, and five out of 15 unclassified monoarthritis or oligoarthritis patients. All these were DR5- (MFI relative to isotype control, 1.2 ± 0.23). Ten out of 10 fibroblast lines from joint replacement surgery or synovectomy specimens of RA were DR5+. All fibroblast lines from the synovium of 10 OA patients were DR5-, as were fibroblasts from the skin of five healthy subjects. DR5+ Sfld cultures underwent apoptosis when treated in vitro with an anti-DR5 antibody, as determined by propidium iodide and annexin V staining. These results suggest that fibroblast-like cells from Sfld of RA are of at least two different lineages. Those expressing DR5 protein share this characteristic with transformed cells and with synovial fibroblasts from RA synovial membrane, and more likely derive from the synovium. The origin of DR5- Sfld fibroblasts remains to be determined. Because all RA patients with DR5- Sfld fibroblasts had active disease, we do not believe that DR5- cells are derived from ‘burnt out’ synovial membrane.

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The active metabolite of leflunomide, A77-1726, increases proliferation of human synovial fibroblasts in presence of IL-1β
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Background Leflunomide is an immunomodulatory agent used for the treatment of rheumatoid arthritis (RA) and is known to inhibit the proliferation of several cell types, including T lymphocytes.
Objectives Because pannus formation due to excessive synoviocyte proliferation is generally associated with the development of RA, we sought to determine the effects of A77-1726, the active metabolite of leflunomide, on synovial fibroblast proliferation.
Methods Cell viability was investigated by measuring at 490 nm the formation of formazan from MTS tetrazolium, and cell proliferation by determination of thymidine incorporation and cell count.
Results Whereas A77-1726 alone had no significant effects on proliferation, it dramatically increased the mitogenic effects of IL-1β. Then we investigated the possible mechanisms of this effect of A77-1726 (i.e. inhibition of DHODH, of tyrosine kinases and COX). The effect was not mediated through inhibition of DHODH because it was not reversed by addition of exogenous uridine. It was not due to inhibition of tyrosine kinases either, because the broad-range inhibitor genistein and the src tyrosine kinase inhibitor PP1 blocked rather than increased IL-1β effects. Finally, although in the absence of IL-1β FGE2 dose-dependently decreased proliferation, it had no effect in the presence of IL-1β, and treatment with indomethacin did not consistently reproduce the effects of A77-1726.
Conclusions The mitogenic effects of A77-1726 in presence of IL-1β may thus be dependent on DHODH and tyrosine kinase inhibition, whereas the role of COX is more uncertain; identification of involved mechanisms may prove useful for the development of related compounds or new therapeutic strategies.
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Protection of inflammation-related bone loss by OPG and TNF-α antibodies through distinct mechanisms in collagen-induced arthritis
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Background Focal and systemic bone loss in rheumatoid arthritis (RA) involves several cytokines such as RANKL, which is secreted by both osteoblasts and activated lymphocytes, and TNF-α. Anti-TNF-α antibodies decrease articular inflammation and bone erosions, but the effect on bone remains to be elucidated. Osteoprotegerin (OPG) inhibits bone resorption.
Objective We conducted the study to evaluate the respective and combined effect of OPG and anti-TNF-α antibodies on inflammation and on bone remodelling.
Methods DBA/1 mice were immunized then treated at the onset of arthritis with OPG-Fc or anti-TNF-α antibodies or with both OPG-Fc + anti-TNF-α antibodies or saline; one group of mice remained untreated (naïve). Deoxypyridinoline changes (ΔD-Pyr) in urine, bone mineral density gain (ΔBMD) at the total body level, and histomorphometric parameters at the femur metaphysis were measured.
Results Anti-TNF-α antibodies but not OPG decreased clinical and histological scores (P < 0.02 versus saline). ΔBMD was lower in saline-treated CIA mice than in naïve mice (P < 0.01), suggesting the effect of inflammation on bone loss. OPG and anti-TNF-α antibodies increased ΔBMD compared with saline (P < 0.001 and P < 0.05, respectively). ΔD-Pyr decreased by 65% with OPG and by 7% with saline (P < 0.001), but by 13% when mice were treated with TNF-α antibodies (NS). Compared with saline, OPG induced increased trabecular (Tb) bone volume (P < 0.02), decreased Tb spacing (P < 0.02) and decreased BFR/BS (P < 0.01). In contrast, the anti-TNF-α antibody treated group showed no significant changes in Tb bone volume and Tb spacing compared with saline, but Tb thickness was greater (P < 0.02), close to that in naïve mice, suggesting a protective effect on bone formation. There was no additive effect of OPG and anti-TNF-α antibodies on any parameter.
Conclusion Administration of OPG and anti-TNF-α antibodies prevented bone loss in CIA mice through distinct mechanisms involving
inhibition of bone resorption and formation. Combinations of both treat-
ments must be further investigated.

63 Repair of local bone erosions and reversal of systemic bone loss upon therapy with anti-TNF in combination with OPG or PTH in TNF-mediated arthritis

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Local bone erosion and systemic bone loss are hallmarks of rheuma-
toid arthritis and cause progressive disability. Tumour necrosis factor (TNF) is a key mediator of arthritis and acts catabolically on bone by stimulating bone resorption and inhibiting bone formation. We hypothe-
sized that the concerted action of anti-TNF, which reduces inflamma-
tion, and of PTH, which stimulates bone formation, or of OPG, which blocks bone resorption, could lead to repair of local bone erosions and reversal of systemic bone loss. To test this, human TNF-transgenic (hTNFtg) mice with established erosive arthritis and systemic bone loss were treated with PTH, OPG and anti-TNF, alone or in combination. Local bone erosions almost fully regressed, upon combined treatment with anti-TNF and PTH and/or OPG, suggesting repair of inflammatory skeletal lesions. In contrast, OPG and anti-TNF alone led to arrest of bone erosions but did not achieve repair. Treatment with PTH alone had no influence on the progression of bone erosions. Local bone ero-
sions showed all signs of new bone formation such as the presence of osteoblasts, osteoid formation and mineralization. Furthermore, sys-
temic bone loss was completely reversed upon combined treatment and this effect was mediated by osteoblast stimulation and osteoclast blockade. In summary, we conclude that local joint destruction and sys-
temic inflammatory bone loss due to TNF can regress, and that repair requires a combined approach by reducing inflammation, blocking bone resorption or stimulating bone formation.

64 Histochemical detection of oxidative stress in synovial tissue: spontaneous reactive oxygen species (ROS) production by rheumatoid arthritis T cells

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Background Oxidative stress is thought to underlie the altered patho-
genic behavior of T cells in rheumatoid arthritis (RA). We recently demonstrated that intracellular signaling by Ras family GTPases leads to ROS production in RA synovial fluid (SF) T cells. However, other cellular sources of ROS may cause oxidative stress in RA synovial tissue (ST).

Objectives The study was conducted to determine whether T cells or other cells spontaneously produce ROS in RA ST, to determine the specificity of T cell ROS production in long-standing RA versus early RA and osteoarthritis (OA) T cells, and to identify signaling pathways producing ROS in RA ST and SF T cells.

Methods ROS production was assessed using ROS-dependent polymer-
zation of diaminobenzidine (DAB) on cryostat sections of ST from patients with long-standing RA, early RA, and OA (n = 10 patients in each group), purified RA patient peripheral blood (PB) and SF mononuclear cells, and purified T cells (n = 4), and oxidation of the dye DCF by FACS analysis of purified RA PB and SF T cells (n = 10), in the absence or presence of pharmacological inhibitors. Cryostat sections were double labelled with DAB and cell-specific antibodies to quantify ROS-producing cells.

Results ROS production was observed in both longstanding and early RA, but not OA ST. ROS production was observed only in T cells (68%) and neutrophils (15%). In RA ST, ROS producing T cells were observed in the synovial sublining and perivascular tissue. Catalase and BAPTA-AM, but not other ROS inhibitors, blocked ROS produc-
tion in ST and SF T cells. Intracellular ROS production is detected in ST of patients with RA but not OA. T cells are the major source of intra-
cellular ROS production in RA ST. Conserved signaling pathways reg-
ulate ROS production in ST, SF and Jurkat T cells. ROS production is observed in recently extravasated T cells, indicating that oxidative stress is an early event affecting T cell function in the RA synovial joint.

65 Immunization with the glycolytic enzyme glucose-6-
phosphate isomerase (GPI) induces peripheral polyarthritis in genetically unaltered mice

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Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology that primarily affects the joints. Recently, a T cell receptor trans-
genic murine model yielded novel insights. K/BxN TCR transgenic mice spontaneously develop an autoimmune arthritis that resembles human RA. Arthritis development depends on the recognition of the ubiqui-
tously expressed glycolytic enzyme GPI by both the transgenic T cells and B lymphocytes. Here we show that immunization with heterologous GPI in adjuvant induces a symmetric polyarthritis of the small distal joints in genetically susceptible mice. The course of GPI-induced arthri-
tis is highly predictable: clinically overt arthritis develops 9–10 days after immunization, reaches its maximum at D15 and then slowly resolves. Histologically, the disease is characterized by early synovitis followed by massive cartilage destruction and erosions of the bones and later resolu-
tion of the inflammation, fibrosis, and repair processes. Although anti-
body titers in susceptible and nonsusceptible mouse strains are high, transfer of purified total IgG of sick mice alone do not transfer disease. Antibodies seem to be a major role since Fc-receptor γ-chain deficient mice develop disease with a much lower frequency and reduced severity than the wild-type mice. Treatment with a depleting monoclonal anti-CD4 antibody completely prevents disease. Depletion of CD4+ T cells during disease leads to a rapid resolution of arthritis. Therefore, CD4+ T cells are important for both the induction of the disease and the effector phase. Thus, GPI-induced arthritis in normal mice bridges the gap between the transgenic K/BxN model and the highly complex human situation, thereby providing a model in which both the induction and effector phase of antigen specific arthritis can be dis-
sected, and preventive and therapeutic strategies evaluated.

66 Differential expression and response to infliximab treatment of specific macrophage subsets in inflammatory synovitis

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Background Myeloid related protein (MRP)8 and MRP14 are expressed in early monocytes, whereas CD163 is marker for mature tissue macrophages.
Objective To analyse macrophage subsets in RA and SpA, and the effect of infliximab on the synovial infiltration of these subsets.

Methods Paired synovium and synovial fluid (SF) were collected in 20 RA and 30 SpA patients. In nine SpA patients, synovium was also obtained after 12 weeks of infliximab treatment. The levels of expression of MRP8, MRP14, CD163 and CD68 were analyzed by immunohistochemistry on frozen sections. Levels of MRP8/MRP14 in SF were determined by ELISA.

Results In inflammatory arthritis, MRP8, MRP14, CD163 and CD68 positive cells were abundant in the lining and sublining layer. The expression of CD68 and CD163 exhibited a cellular pattern. The MRP8 and MRP14 expression was characterized by a cellular staining, with in some samples also an extracellular and perivascular pattern. Comparing RA and SpA, CD68-positive macrophages were equally present (lining $P=0.463$, sublining $P=0.235$), whereas MRP8 expression was higher in the lining of RA synovium ($P=0.025$) and CD163-positive cells were more prominent in SpA synovium (lining $P<0.001$, sublining $P=0.017$). No differences for MRP8, MRP14, CD163 or CD68 were seen between the SpA subgroups. Confirming that these markers identify different macrophage subsets, the expression of MRP8 and MRP14 was more reduced after infliximab than CD163 (MRP8: lining $P=0.038$, sublining $P=0.023$; MRP14: lining $P=0.088$, sublining $P=0.036$; CD163: lining $P=0.119$, sublining $P=0.126$). Local secretion of soluble MRP8/MRP14 was evidenced by high SF levels (RA 24.530 ng/ml, SpA 4152 ng/ml), which were higher than in serum (RA 20-fold, SpA 6-fold).

Conclusion MRP8/MRP14 and CD163 identify different macrophage subsets as evidenced by the differential expression in RA versus SpA, and by the differential effect of infliximab on the synovial expression of these markers.

Genomics and signal transduction

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Monocyte-specific gene signatures in rheumatoid arthritis

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Background In rheumatoid arthritis (RA), monocytes are of central importance in the chronic inflammatory immune response. These cells may display diagnostic markers and provide insight into pathophysiological pathways in RA.

Objective To identify characteristic expression patterns in rheumatoid arthritis (RA), highly purified peripheral blood monocytes were investigated.

Methods Monocytes from five normal donors (ND) and six patients with active RA were purified by erythrocyte lysis and CD14-positive selection at constant low temperature ($4^\circ$C) conditions to minimize in vitro induced artefacts. Gene expression was detected by Affymetrix HG U133A hybridization. Bioinformatic analysis included MASS.0 image analysis, statistical methods ($t$-test; Mann-Whitney) and cluster analysis (genes@work).

Results Array analysis revealed about 50% present calls in ND or RA monocytes. MASS.0 comparison expression analysis revealed 605 genes that were significantly changed in more than 50% of all RA versus ND pair-wise comparisons. Statistical methods revealed that 260 of these genes were significantly different ($P<0.05$). Selection and reduction by fold changes above 1.6 and statistical methods reduced the number of differentially expressed genes to 152. This set of genes is implicated in cellular processes that are involved in cell communication (cytokines, chemokines and their receptors), intracellular signalling, transcription, defense response, apoptosis and heat shock responses. Hierarchical cluster analysis based on these selected genes separated into two branches, one for ND and one for RA patients.

Conclusion Gene expression profiling of separated peripheral blood monocytes identified a characteristic gene pattern that is involved in the activation of monocytes, and clearly indicates an inflammatory process in RA. These results will be compared with expression profiles in other inflammatory rheumatic diseases and further analyzed for dominant pathophysiological pathways in a search for new candidates for targeted therapy in RA.
lager–pannus junction. Furthermore, therapeutic neutralization of molecules produced by activated MF lead to clinical improvement in RA, and circulating monocytes (MO) of the peripheral blood in patients with RA spontaneously express proinflammatory genes (IL-1β, IL-6, TNF).

**Methods** A custom RA-MO cDNA microarray was generated using differentially expressed genes obtained from gene subtraction and from comparative whole genome wide U133A analysis in normal donors, active and anti-TNF-α treated RA patients. Genes were selected using MAS 5.0, multtest and PAM. The custom microarray consists of 313 genes including guide dots, and positive (housekeeping genes and spike controls) and negative controls for image and statistical analysis. Each probe was spotted in 16 replicates.

**Results** The RA-MO chipset-II was validated using the following: non-stimulated and LPS, PMA, Vit.D3+LPS, PMA+LPS stimulated U937 cells; nonstimulated and LPS stimulated healthy donor MO; MO from normal donors (n = 3) and RA patients before and during anti-TNF-α treatment (n = 5 each); and synovial tissue from normal individuals (n = 2) and RA patients (n = 2). Not only LPS/PMA regulated genes but also RA specific and anti-TNF-α regulated genes were validated. In addition, we could clarify whether these genes are differentially transcribed only in MO or whether they can also be found in RA tissue MF. Our data indicate a high degree of reproducibility that is sufficient for diagnostic applications and therapy monitoring.

**Conclusion** The RA-MO chipset-II microarray is competitive and flexible for enlargement of the number of genes. The current gene selection will contribute to validating the role of monocytes in disease activity, to therapeutic interventions, and may improve the knowledge on the regulation of pathways in activated monocytes in chronic inflammation.

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**Reporter cell lines for real-time imaging of NF-κB activation in the arthritic joint**

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**Background** Inadequate functioning of NF-κB is implicated in many diseases, such as rheumatoid arthritis (RA), and increased activation of NF-κB in synovial tissue precedes the clinical manifestation of experimental arthritis.

**Objective** The aim of the study was to develop NF-κB reporter cell lines that can be used for real-time evaluation of NF-κB activation in knee joints during experimental arthritis in living animals.

**Methods** For determination of NF-κB transcription activity, an eukaryotic expression vector was created containing five tandemly arranged NF-κB binding sites (5’-GGGACCTTCC-3’) regulating luciferase expression. Cell lines were selected on stable incorporation of 5’×NF-κB luciferase and responsiveness was tested toward NF-κB inducing stimuli.

**Results** These cell lines, derived from fibroblasts, chondrocytes and thymocytes, showed good cytokine responsiveness toward IL-1, IL-17, IL-18, and TNF. We showed that these cells could also be used in vitro inhibition assays using cytokine protein antagonist (e.g. IL-1Ra and IL-18BPc) or RNA interference. By transplanting these cells into HLA matched recipients we were able to image in real time NF-κB activation in knee joints of mice with IL-1- or SCW-induced inflammation using a cooled-charge-coupled device (CCCD) camera. Interestingly, NF-κB activation was also activated at sites remote from the monokine or SCW injection sites. Synovial mRNA revealed a striking upregulation of IL-1 in both SCW challenged and contralateral knee joint. This effect was confirmed by pretreatment of mice with neutralizing anti-IL-1 Abs, which markedly prevented the NF-κB activation in the contralateral naive joints.

**Conclusion** These reporter cell lines can be a valuable tool to study the role of NF-κB activation in arthritis and to evaluate cytokine/NF-κB based inhibition therapies. Furthermore, we show that local induction of experimental arthritis results in contralateral NF-κB activation, which is dependent on IL-1.

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**Differential regulation of mitogen-activated protein kinases by thymosin β4 sulphoxide (Tb4SO)**

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**Background** Thymosin β4 (Tb4) is a 4.9 kDa ubiquitously expressed intracellular peptide known to sequester G-actin, with a growing number of extracellular biological functions. We have shown that oxidation of its single methionine residue at position 6 (Tb4 sulphoxide [Tb4SO]) gives the peptide potent anti-inflammatory properties. In addition, Tb4 promotes wound healing in various *in vitro* and *in vivo* models. The mechanism by which Tb4SO exerts its extracellular biological functions is unknown. Its cell receptor remains unidentified and intracellular signalling consequences of Tb4SO require elucidation.

**Objective** The study was conducted to investigate the intracellular signalling consequences of the addition of Tb4SO to various cell types, specifically MAP kinase activation.

**Methods** Cell types used were treated with Tb4SO and lysed using RIP@ buffer. Lysates were run on SDS PAGE, transferred to PVDF membrane and probed with antiphospho ERK1/2, p38, JNK1/2 or AKT antibodies. Membranes were then stripped and reprobed with pan-antibodies to allow normalization of samples.

**Results** Tb4SO induced the phosphorylation of ERK1/2 in peripheral blood-derived monocytes in dose-dependent manner. This was inhibited by addition of U0126, an inhibitor of the upstream activator of ERK1/2, implicating ERK1/2 in Tb4SO-mediated functions in monocytes. Neither p38 MAPK nor JNK1/2 were activated in peripheral blood monocytes. In peripheral blood-derived macrophages, Tb4SO induced rapid and marked activation p38 MAPK, pAkt and ERK1/2, but not of JNK (p-JNK1/2). Interestingly, only JNK1 was activated by Tb4SO in HeLa cells, and this occurred in a delayed (after 15 min) and transient manner. Short-term activation of JNKs has previously been shown to be involved in the upregulation of apoptosis. Preliminary studies have shown an acceleration in neutrophil but not monocyte/macrophage apoptosis in response to Tb4SO.

**Conclusions** These observations suggest that Tb4SO activates MAPKs and that activation is dependent on cell type, which may in turn regulate differential functions in these different cell types.

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**Polymorphism at the position −308 of TNF-α gene influences outcome of infliximab therapy in rheumatoid arthritis**

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**Background** Not all rheumatoid arthritis (RA) patients respond well to the standard infliximab regimen. No clinical or biological factor that would allow one to predict response has yet been identified.

**Objective** The aim of the study was to test whether the −308 G/A polymorphism in the promoter of the TNF-α gene and the −1082 G/A polymorphism in the promoter of the IL-10 gene influence response to infliximab therapy in patients with RA.

**Methods** We genotyped 85 RA patients for −308TNFα polymorphism by PCR and subdivided them into group A (A/A or A/G genotypes) and group B (G/G genotypes). We compared clinical response to infliximab treatment between the two groups after four infusions, using the DAS28 index.

**Results** We found that 47.4% of patients in group A and 79.3% of patients in group B had DAS28 index improvement greater than 1.2 (P = 0.0075) and that the average DAS28 improvement was 1.12 in S23
group A and 2.08 in group B \( (P = 0.05) \). IL-10 polymorphism did not allow us to discriminate between good and poor responders.

**Conclusion** These data suggest that the –308 A/G and the –308 A/A TNF-\(\alpha\) genotypes predict poor response to infliximab therapy in RA. –308TNF\(\alpha\)Falla genotyping might represent an easy tool with which to predict response to infliximab treatment. Conversely, the IL-10 polymorphism did not appear to be useful.

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**Unraveling complexity of rheumatoid synovial gene expression by comparison with purified leukocyte profiles**

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**Background** Gene array analyses reflect the molecular complexity in rheumatoid synovitis. Insufficient knowledge about the majority of differentially regulated genes hampers adequate interpretation.

**Objective** To unravel this complexity, cell type specific expression profiles were applied as baseline information for comparison.

**Methods** Gene expression profiles were determined by Affymetrix HG-U133A array hybridization of synovial tissues, sorted blood monocytes, granulocytes, CD4 and CD8 T cells from up to 10 patients with rheumatoid arthritis (RA) and 10 normal donors (ND). Primary analysis was performed using MA5.0. Tissues were scored according to histological standards (Krenn, Pathol Res Pract 2002).

**Results** Compared with ND synovium, 145 genes were differentially regulated in all, and up to 2681 genes in at least 50% of RA-ND pairwise comparisons. Marker genes of isolated cell populations were defined by absence in more than 70% of other populations and normal synovial tissue, defining 59, 25 or 110 genes for granulocytes, monocytes or T cells, respectively. Of these markers, 1, 6 or 22 were identified in more than 50% of RA-ND comparisons for each cell type, respectively. This reflects the altered cellular composition in synovitis. Excluding all genes present in normal tissue or any of the purified populations revealed immunoglobulins as markers of B-cell infiltration.

**Conclusion** This initial analysis of our approach substantially improved the quality of array interpretation and allows identification of tissue associated gene regulation. It helps to identify markers shared between blood and tissue and may provide candidates for disease classification and activity scoring.

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**B lymphocytes infiltrating Wegener's granuloma: the immunoglobulin VH gene repertoire from granulomatous tissues displays an antigen-driven maturation and suggests a microbial trigger**

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**Background** In Wegener’s granulomatosis (WG) the development of a generalized vasculitis is based on strong expression of proteinase 3 (PR3) on the surface of neutrophil granulocytes and the appearance of circulating high-affinity anti-PR3 autoantibodies (PR3-ANCA) suggesting an antigen-driven B lymphocyte maturation.

**Objective** In order to analyze mechanisms relevant to the induction of ANCA, we compared the immunoglobulin heavy chain (VH) gene repertoire in biopsy specimen of granulomas from two cases of ANCA-negative localized WG and from two cases of ANCA-positive generalized WG with a healthy control.

**Methods** Frozen tissue samples were stained for B lymphocytes by anti-CD20 APAAP. DNA was prepared, purified and subjected to a PCR with individual VH primers matching to the families VH1–VH6 and a JH consensus primer. After bacterial subcloning PCR products were sequenced and characterized for gene usage and mutational pattern.

**Results** Immunohistology revealed lymphoid infiltrates within typical WG granulomas containing more than 30% B cells, which is suggestive of germinal center formation (Fig. 1). One hundred and fifty differently rearranged VH genes from WG tissues were characterized and compared with 84 VH genes from peripheral blood of a healthy donor. The mutation frequency as well as the ratio of amino acid replacement to silent mutations indicate an antigen-driven selection of antibodies within such germinal center-like regions of the granuloma in WG and differ significantly from the healthy control. The VH genes in WG revealed both striking similarities to published PR3-ANCA-encoding genes as well as to VH genes from Staphylococcus aureus superantigen- and B. cereus B cells. Furthermore, 50% of mutations within the binding-site coding regions led to negatively charged amino acids that favour affinity to the positively charged PR3.

**Conclusion** In WG a subset of autoreactive PR3-producing B cells may be induced to produce PR3-ANCA within granulomatous lesions after Staphylococcus aureus B-cell superantigen stimulation. Selected B cells undergo affinity maturation and differentiate into plasma cells that produce high levels of circulating ANCs, which are involved in the pathogenesis of the systemic vasculitis.

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**Toll-like receptor 4 polymorphism influences susceptibility to but not severity and outcome of rheumatoid arthritis**

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**Introduction** Toll-like receptors (TLRs) play an important role in the innate and adaptive immune response. TLR4 is the most thoroughly studied member of this receptor family and interacts with endogenous
The aim of the present study was to analyze the relationship between the TLR4 Asp299Gly (896A>G) polymorphism and RA susceptibility, disease phenotype and outcome over a 6-year period of the disease.

Methods Genotyping was performed on RA patients from an early RA inception cohort. Healthy controls consisted of healthy blood donors. To assess the influence of the TLR4 variant on disease phenotype, course and outcome, we compared prospectively collected disease activity and outcome parameters of patients with and without the variant TLR4 allele. TLR4 genotyping of the A>G polymorphism at position 896 of the TLR4 gene was performed using RFLP.

Results In 282 RA patients and 314 healthy individuals we found that the frequency of the TLR4 Asp299Gly polymorphism was significantly lower among RA patients (10.8%) than among controls (17.2%; P = 0.02). To analyze potential differences in disease phenotype, severity and outcome 30 RA patients heterozygous for the TLR4 variant were compared with 252 RA patients possessing two wild-type alleles. Apart from the higher DAS at baseline (6.1 versus 5.4; P = 0.01), no other differences in phenotype, severity or outcome of RA were detected between both groups.

Discussion Here we demonstrate a link between RA disease susceptibility and the TLR4 polymorphism in a large set of RA patients and healthy individuals. Furthermore, we show that the TLR4 polymorphism might be important in the onset of disease but does not seem to play a role in disease outcome.

76 Radiological sacroiliitis is linked with CARD15 gene polymorphisms in patients with Crohn's disease

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Background Sacroiliitis (SI) is a common extrarticular manifestation of Crohn's disease (CD). In contrast to idiopathic ankylosing spondylitis (AS), its association with HLA-B27 is less evident. Mutations in the CARD15 gene are recently identified as susceptibility genes for CD. This study aimed to identify whether the CARD15 gene encodes the intracellular NOD2 receptor and plays an important role in innate immunity. Studies on associated disease phenotypes revealed a predisposition for ileal localization, early onset of disease, familial cases and fibrotenosising disease. We recently described a higher prevalence of CARD15 variants in a subgroup of spondyloarthropathy patients with chronic gut inflammation, which are prone to evolution to CD.

Background The present study evaluated whether CARD15 polymorphisms are associated with sacroiliitis in patients with CD.

Methods One hundred and two consecutive CD patients were clinically evaluated by a rheumatologist. Radiographs of sacroiliac joints were performed and blindly scored by two investigators. RFLP-PCR technique was used to genotype all patients for three single nucleotide polymorphisms in the CARD15 gene. Every SNP was verified by direct sequencing. HLA-B27 phenotype was determined.

Results Radiological evidence of SI with or without AS was present in 23 patients (23%). Of patients with SI 78% carried a CARD15 variant, versus 48% of patients without SI (P = 0.011; OR 3.8, 95% CI 1.3–11.5). A logistic regression analysis with SI as dependent variable and CARD15, HLA-B27, familial cases, need for resection surgery, age of diagnosis and ileal disease as covariates showed that only carriage of CARD15 variants was a significant predictor of SI (P = 0.035).

Conclusion This result suggests that carriage of CARD15 variants is a genetic marker for CD related SI, independent of HLA-B27 or other CARD15 related phenotypes.

77 Fibroblast-like synoviocytes derived from patients with rheumatoid arthritis show the imprint of synovial tissue heterogeneity: evidence for the existence of distinctive pathways relevant to disease

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The molecular pathogenesis of rheumatoid arthritis (RA) is still poorly understood and its clinical course can vary widely. In a recent report we noted heterogeneity in global gene expression signatures between synovial tissue specimens from different patients with RA. The results of the present study support the view that clinically diagnosed RA is a heterogeneous disease that is featured not only at the whole synovial tissue level but also at the level of FLS cultured from those tissues.

Gene therapy

78 Host immune responses to salivary gland administration of recombinant adeno-associated virus serotype 2 vectors

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Background Salivary glands (SGs) provide a novel target site at which gene transfer can be accomplished in a minimally invasive manner [1]. SGs are also capable of producing large amounts of proteins, and human SGs are well encapsulated, a circumstance likely to minimize the undesirable access of administered vectors and transgenes to other tissues [2]. Previous studies have indicated that intravenous, intramuscular and intranasal administration of rAAV2 vectors induce relatively mild host immune responses.

Objective The aim of the study was to examine the effects on immune responsiveness of rAAV2 vector delivery into SGs.

Methods The main excretory ducts of the submandibular glands of Balb/c mice were cannulated and vectors administered by retrograde...
infusion [1]. On day 0 we delivered a rAAV2 encoding β-galactosidase or saline (control) to adult mice. On day 28, we administered a second rAAV2 vector encoding either human erythropoietin (Epo) or human growth hormone to one-third of each group. Immune activities were evaluated in saliva, serum, SGs and spleens collected at different times.

Results Vector delivery had little effect on salivary flow rates at all time points studied. Histological examination of SGs on day 1 did not indicate any significant inflammatory cell infiltration due to rAAV2 vectors. Ex vivo stimulation, with rAAV2, of splenocytes on days 28 and 56 resulted in elevated interferon-γ levels in culture media from rAAV2-administered mice, but not from control mice. Significant titers of neutralizing antibodies to rAAV2 were detected in serum, with generally lower levels found in saliva. We were unable to observe transduction of SG cells by rAAV2Epo in mice previously infected with rAAV2LacZ (i.e. no elevations in hematocrit [Hct] and serum Epo levels were seen in the virus re-administration group). Conversely, we found significant elevations in Hct and serum Epo levels in mice previously administered saline.

Conclusion We conclude that after a single administration of rAAV2 to murine SGs there is no significant innate immune response. However, we observe a modest adaptive immune response that abrogates the efficacy of additional rAAV2administration.

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References

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Switching of serotype can improve local AAV-mediated gene therapy in RA

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Objective Adeno-associated virus (AAV) is a very promising vector for gene therapy in RA. Although an increasing number of AAV serotypes have been identified, all studies so far have been performed with serotype 2. The aim of this study was to compare the transduction efficiency of five different AAV serotypes (AAV1–AAV5) encoding reporter genes, the murine secreted alkaline phosphatase (mSEAP) or the E. coli β-galactosidase (beta-Gal), in two different animal models of arthritis.

Methods AAV1–AAV5 containing the gene for beta-Gal were injected into the right ankle joints of rats with adjuvant arthritis (AA) on day 12 after adjuvant immunization. Joints were collected 2 weeks after injection. The number of viral genomic copies in the joint was determined by qPCR. For detection of LacZ transcription in joints and organs, RT-PCR was used. Beta-Gal expression was analyzed by direct in situ staining of frozen sections, quantified by digital image analysis. In mice with collagen-induced arthritis (CIA), AAV1, 2 and 5 constructs encoding the mSEAP gene were injected into the left knee joint 32 days following arthritis induction. Transgene expression was analyzed by chemiluminescence in sera and culture medium conditioned by the joint tissues at different time points.

Results The greatest number of viral genomic copies was detected using AAV5. This was confirmed by in situ beta-Gal staining. RT-PCR proved the presence of LacZ mRNA in injected joints, but not in any of the organs tested. In the CIA mice model AAV5 also had the best transduction efficiency. Transgene expression was detectable in sera and patellae 1 week after joint injection, increased over time, and remained at plateau levels for at least 1 month.

Conclusion In vivo gene transfer with AAV5 is far more efficient than with other serotypes. Local AAV-mediated gene therapy in RA could be improved by using AAV5.

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Suppressor of cytokine signaling (SOCS)-3 gene transfer protects against collagen-induced arthritis

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Background Rheumatoid arthritis is characterized by an acute phase response of the liver. IL-6 is produced in the inflamed joint and high levels are found in the circulation. IL-6 is a potent inducer of acute-phase proteins (APP) in hepatocytes via its signal transducer gp130 by the activation of the signal transducer and activator of transcription (STAT)-3.

Objective The aim of the study was to determine the effect of adenoviral gene transfer of SOCS-3, the inhibitor of STAT3 activation, on collagen-induced arthritis (CIA).

Methods Livers were dissected, and activation of STATs was analyzed by Western blot and gene expression by RT-PCR. A replication incompetent adenovirus containing the gene of murine SOCS-3 was constructed. Mice received 3 × 106IU of Ad5.CMV.mSOCS3 or control viruses intravenously before onset of arthritis. The effect of SOCS gene transfer on liver function was studied in zymosan-induced gonarthrosis (ZIA) and the therapeutic effect in CIA.

Results Induction of ZIA in the knee joints of mice caused a rapid (within 5 hours) activation of STAT3 but not STAT1 in the liver. The activation of STAT3 was IL-6 dependent, as shown using IL-6 gene knockout mice. Intravenous injection of Ad5.CMV.mSOCS3 24 hours before induction of ZIA prevented STAT3 activation and markedly reduced serum amyloid A (SAA)-1 gene expression in the liver. Control virus treatment did not inhibit STAT3 activation in the liver. Overexpression of SOCS-3 did not affect Erk activation, confirming selectivity for the STAT signaling pathway. Treatment of DBA1/J mice with Ad5.CMV.mSOCS3 virus after immunization ameliorated CIA, and reduced serum TNF-α levels accompanied this effect. SOCS-3 treatment had no effect on the circulating levels of total IgG, IgG2a and IgG1 anti-collagen type II antibodies.

Conclusion Inhibition of STAT-mediated signaling via SOCS-3 adenoviral gene transfer in the liver markedly ameliorated CIA. This suggests that the IL-6 mediated liver response modulates the development of arthritis.

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IL-22 gene transfer in collagen-induced arthritis

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Background and Objective Novel cytokines have been identified by structural homology to IL-10. Among them, IL-22 showed a 25% amino acid identity with IL-10 and shared a common receptor β-subunit. The expression profile suggested involvement in the immune response in vivo. The present work evaluates the role of IL-22 in arthritis, examining the effects of systemic murine IL-22 adenoviral-mediated overexpression (Ad-mIL-22) in the collagen-induced arthritis (CIA) DBA1 mouse model.
Method Human rheumatoid arthritis (RA) synovial fibroblast (SF) were infected in vitro with increasing doses of Ad-mIL-22 (0-25 MOI). Cytokine secretion was assessed in supernatant using a specific ELISA. Therapeutic efficiency of mIL-22 overexpression achieved by intravenous injection of Ad-mIL22 (5 × 10⁹ pfu) after the onset of CIA was assessed. A group injected with a nonrelevant adenoviral vector (Ad-LacZ2) was used as a control. Radiological and histological analyses were performed at day 47. Systemic expression of mIL-22 was assessed by ELISA.

Results After confirmation that human RA-SF infected with increasing doses of Ad-mIL-22 expressed high levels of mIL-22 transgene, we demonstrated that the intravenous injection of Ad-mIL-22 in DBA1 mice significantly reduced clinical, radiological and histological scores. By the end of the experiment, the number of arthritic paws was decreased by 70% in the Ad-mIL-22-treated group as compared with controls (P < 0.0001). Ad-mIL-22 resulted in the complete protection as compared with control vector up to 20 days after treatment. Maximal paw widths reached during the course of the disease were, respectively, 1.95 ± 0.08 versus 2.76 ± 0.60 mm (P = 0.024). Both radiological and histological scores were found to correlate well with clinical observations. Systemic levels of mIL-22 reached 14.2 ± 9.5 ng/ml 4 days after gene transfer, then decreased to 2.4 ± 0.9 ng/ml by day 8 and remained stable until day 21.

Conclusion Our data support that IL-22 may have antiarthritic properties. The molecular mechanisms responsible for this observation are under investigation.

Nonviral gene therapy by electrotransfer of hTNF-α soluble receptor variants: application to the treatment of collagen arthritids

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Objectives Electrotransfer (ET) was used to administer transgenes encoding three hTNF-α soluble receptor-I variants. Electrotransfer parameters and therapeutic effect in collagen-induced arthritis (CIA) in mice were studied.

Methods Plasmids pCOR(hTNFRα1), pCOR(hTNFRα2), encoding a monomeric, a chimeric or a dimeric form of hTNFRβ, respectively, were used. Electrotransfer was performed by plasmids injection and electric pulses in muscle. hTNFRβ concentrations were determined by ELISA. PCR was performed on genomic DNA from various mouse organs to detect the plasmid. CIA was induced by immunization of DBA/1 mice with collagen II in adjuvant.

Results ET of the three plasmids (1–15 µg) allowed hTNFRβ production in sera and muscle after 10 days. This expression was dependent on the dose of plasmid. Local expression in the muscle lasted for at least 6 months. Systemic expression in the serum was detectable right after the electrotransfer. hTNFRβ concentrations were determined by ELISA. PCR was performed on genomic DNA from various mouse organs to detect the plasmid. CIA was induced by immunization of DBA/1 mice with collagen II in adjuvant.

Conclusion Intramuscular ET of plasmids encoding the three forms of hTNFRβ leads to a long-term secretion of hTNFRβ in vivo. CIA is efficiently inhibited when ET of plasmids encoding either the chimera or the dimeric hTNFRβ was performed at the onset of the disease.

Application of regulatable vectors in gene therapy of experimental arthritis

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Background Gene therapy studies in experimental models of arthritis have demonstrated effective treatment with a variety of immunosuppressive and proapoptotic genes using in vivo and ex vivo strategies. Regardless of which approaches are developed for clinical application of gene therapy, we believe that it is imperative that expression of a therapeutic gene is regulated so that levels of expression are controlled and can be downregulated in the event of adverse effects.

Objective We previously constructed an autoregulatory self-contained plasmid vector pGTRTL in which gene expression is induced with doxycycline (Dox). Expression kinetics of luciferase from this vector have been characterized in vitro. In this investigation we examined the dynamics of luciferase expression from pGTRTL delivered in vivo to DBA/1 mice. Therapeutic effect of a small dimeric TNFR2 molecule (dTNFR) expressed constitutively from the vector pcdTNFR and regulated from the vector pGTRTL was compared in DBA/1 mice with established collagen-induced arthritis (CIA).

Methods Plasmid DNA was administered to naïve or arthritic mice by intramuscular injection combined with electroporation. Gene expression from regulated vectors was induced with Dox administered in drinking water. The degree of arthritis was scored every 2–3 days, and at the end of the experiment serum immunoglobulin levels and cytokine release from lymph node cells was measured.

Results The results show efficient regulation of luciferase expression from pGTRTL in vivo. Expression of dTNFR from constitutive or regulated expression vectors in mice with established CIA was therapeutic (Fig. 1), but was dependent upon the level of disease activity when treatment was initiated.

Figure 1

Progression of CIA in mice treated with dTNFR expressed constitutively from pcdTNFR (a) and induced from pGTRTL (b).

Conclusion Improved pharmacologically regulated vectors could be developed for clinical application. There is also scope to utilize disease-responsive vectors that contain DNA binding motifs that respond to transcription factors upregulated in arthritic joints.

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Novel therapies

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Long-term immune reconstitution in patients treated with autologous stem cell transplantation for refractory autoimmune diseases

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Objectives We performed here a detailed analysis of the newly developing immune systems in patients treated with immune ablation and subsequent autologous stem cell transplantation (ASCT) for severe autoimmune diseases.

Methods Peripheral blood lymphocytes were analyzed using flow cytometry, including assessment of TCR-Vβ repertoire of Th cells. Thymic activity was determined by measuring T-cell receptor excision circles (TRECs) in distinct peripheral blood Th cell subsets by quantitative RT-PCR analysis.

Results Twelve patients, with median follow up of 40 months, were included in the trial thus far: polychondritis (n = 1), systemic lupus erythematosus (SLE; n = 6), systemic sclerosis (SSc; n = 3), panniculitis (n = 1) and MS (n = 1). Clinical remission has been achieved in seven patients: polychondritis (n = 1), SLE (n = 5) and MS (n = 1). We observed progress of disease in patients with SSc and panniculitis. In one patient SLE reactivated 17 months after ASCT. In all responding patients no autoreactive Th cells could be detected, and serological remission has been achieved. Lymphocyte compartments reconstituted functionally in all patients as shown by the reappearance and persistence of naïve T cells with high levels of TRECs and restored diversity of the T cell receptor repertoire. Reconstituting B cells were found to be of naïve phenotype. Although we observed similar kinetics of lymphocyte reconstitution in the nonresponding SSc patients, autoantibody titres (ANA, SCL70) were not affected by conditioning.

Conclusion The newly generated immune system in responding patients is tolerant to rheumatic autoantigens and able to react to pathogens. We conclude that in successfully treated patients autoreactive memory and effector lymphocytes have been eliminated efficiently. Our data demonstrate that ASCT can induce stable long-term clinical and serological remission in patients with severe, standard-therapy refractory autoimmune diseases.

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Zoledronic acid protects from local and systemic bone loss in TNF-mediated arthritis

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Objective Increased osteoclast activity is a key factor for bone loss in rheumatoid arthritis (RA). This suggests that osteoclast-targeted therapies could effectively prevent skeletal damage in RA. Zoledronic acid (ZA) is one of the most potent agents to block osteoclast function. We therefore investigated whether ZA can inhibit inflammatory bone loss.

Methods Human TNF transgenic (hTNFtg) mice, which develop severe destructive arthritis as well as osteoporosis, were treated with phosphate-buffered saline, single or repeated doses of ZA, calcitonin or anti-TNF at the onset of arthritis.

Results Synovial inflammation was not affected by ZA. In contrast, bone erosion was retarded by single administration (–60%) and almost completely blocked by repeated administration (–95%) of ZA. Cartilage damage was partly inhibited (–40%), and synovial osteoclast counts were significantly reduced upon ZA treatment. Systemic bone mass dramatically increased in hTNFtg mice upon ZA administration, which was due to an increase of trabecular number and connectivity. In addition, bone resorption parameters were significantly lowered after ZA. Calcitonin had no effect on synovial inflammation, bone erosions, cartilage damage, or systemic bone mass. Anti-TNF entirely blocked synovial inflammation, bone erosion, synovial osteoclast formation and cartilage damage, but had only minor effects on systemic bone mass.

Conclusion ZA appears an effective tool with which to protect bone from arthritic damage. In addition to anti-inflammatory drug therapy, modern bisphosphonates are promising candidates to maintain joint integrity and to reverse systemic bone loss in arthritis.

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Abatacept (CTLA4Ig) treatment increases the remission rate in rheumatoid arthritis patients refractory to methotrexate treatment

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Background Effective amelioration of symptoms and induction of remission are goals in treatment of rheumatoid arthritis (RA).

Objectives Data from a Phase II study for RA treatment with abatacept, a selective co-stimulation modulator, showing induction of remission (DAS-28 score <2.6) are presented.

Methods Patients on background methotrexate (MTX) who met ACR criteria for active RA with ≥10 swollen joints (66 joint count) and ≥12 tender joints (68 joint count) were randomly assigned to receive 10 mg/kg abatacept (n = 115) or placebo (n = 119) treatment for 1 year. DAS-28 scores and serum cytokine levels were assessed at days 1, 90, 180 and 360.

Results Abatacept-treated patients showed a progressive increase in remission rates up to 1 year (analysis not prespecified) compared with placebo (P < 0.001; Fig. 1). Abatacept treatment also decreased serum levels of proinflammatory cytokines. In particular, levels of serum IL-6, a multifunctional cytokine that contributes both to acute phase response and to pathological B cell activation, were reduced by 67% at 180 days and by 73% at 360 days (P < 0.05). Placebo-treated patients showed no reduction. Abatacept was generally safe and well tolerated.

Figure 1

Abatacept increases the remission rate in RA patients refractory to MTX treatment. Means and 95% confidence intervals are shown.
Conclusions In patients with active RA who were receiving MTX, abatacept treatment significantly improved RA symptoms and produced a progressive increase in remission rates for over one-third of the treatment group, which was sustained at 1 year. In addition, abatacept decreased serum IL-6 levels. The results of this phase II study suggest that abatacept may have potential as therapy for patients with active RA despite MTX treatment.

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87 Multidrug resistance proteins and DMARD efficacy in rheumatoid arthritis

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Background Uptregulation of drug efflux pumps belonging to the family of ATP-binding cassette (ABC) transporters can confer loss of efficacy/resistance to cytostatic drugs, a phenotype referred to as multidrug resistance (MDR). In clinical rheumatology practice, loss of efficacy of disease-modifying antirheumatic drugs (DMARDs) is observed following long-term treatment of rheumatoid arthritis (RA) patients. Whether loss of efficacy/resistance to DMARDs and cytostatic drugs share common molecular mechanisms is not known.

Objective The aim of this study was to delineate the potential role of MDR proteins in the loss of efficacy/resistance to DMARDs.

Methods In vitro model systems of human T cells and monocytic/macrophage cell lines were used to provoke resistance to the DMARD sulfasalazine (SSZ). SSZ-resistant cell lines, as well as macrophages from RA patients and healthy controls, were characterized for expression of MDR proteins.

Results Development of SSZ resistance in T cells and monocytic/macrophage cell lines was accompanied by a marked induction of the MDR protein ABCG2. Of note, SSZ-resistant cells displayed cross-resistance to methotrexate, but showed enhanced sensitivity to glucocorticoids (prednisone, dexamethasone). Immunohistochemical studies revealed increased expression of MDR proteins in macrophages from RA patients as compared with controls.

Conclusion These results suggest that MDR proteins, originally identified for their role in resistance to cytostatic drugs, could also be involved in DMARD resistance [1]. Beyond this, resistance to one particular DMARD can influence the sensitivity to other antirheumatic drugs.

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88 Impact of cellular therapies on autoreactive, long-lived plasma cells in autoimmune diseases (AID)

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According to the current view, chronic AID are driven by the continuous activation of autoreactive B and T lymphocytes. However, despite the use of potent immunosuppressive drugs, these patients exhibit continued autoantibody production, which often contributes to progression of the AID. To elucidate this problem, we analyzed the lifespan of cells secreting (auto)antibodies (Ab) in NZB/W mice. The number of Ab-secreting cells in splenic tissue increased from ages 1 to 5 months and became stable thereafter. Later, some 60% of the total Ab-secreting cell compartment consisted of dividing, short-lived plasmablasts (SLPB), whereas 40% consisted of nondividing, long-lived plasma cells (LLPC) with a half-life of more than 6 months. Although high-dose cyclophosphamide eliminated SLPB, LLPC survived and continued to produce (auto)Ab. This explains why treatment with cyclophosphamide (CY) does not result in the complete disappearance of antinuclear Ab. Pathogenic anti-dsDNA Ab usually vanish after this treatment, indicating that they are generated by proliferating SLPB. However, anti-dsDNA Ab can be resistant to CY in refractory SLE.

We treated six refractory SLE patients by autologous stem cell transplantation (ASCT) using CY and ATG as the mobilization regimen. ASCT was performed using purified CD34+ stem cells. The patients have now been followed for 5–70 months. Initially, persistent anti-dsDNA disappeared within 3 months. Likewise, the ANA became negative except in one case. That patient continued to exhibit anti-Ro/SSA and anti-La/SSB, the titers of which decreased continuously and were undetectable after 17 months. This emphasizes the enormous lifespan of residual plasma cells after interrupting the supply of new cells from the dividing SLPB compartment.

In conclusion, the aforementioned ASCT protocol can lead to depletion of autoreactive LLPC. Since ASCT may not always be able to eliminate autoreactive LLPC, these cells are potential targets for autoimmune disease therapy.

89 Addition of cyclosporine-A (Neoral®) in patients on anti-TNF and methotrexate therapy improves disease activity: an open-label, pilot study

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Background Combining anticytokine treatment with a T-cell directed agent such as cyclosporine A (CsA) may rescue patients refractory to anti-TNF treatment. We performed an open-label, pilot study to explore the safety and initial efficacy of a triple therapeutic regimen with anti-TNF, methotrexate (MTX) and CsA.

Methods Nineteen patients with treatment-resistant RA have been enrolled. Patients had considerable residual disease activity (mean DAS-28 6.8, 4.5–8.6), although they had received a mean of 16.8 infliximab infusions (range 6–23) and adequate dose adjustments of both infliximab (mean dose 4.2 mg/kg qw) and MTX (mean dose 17.1 mg/week). CsA (mean dose of 2.7 mg/kg per day, range 1.6–3.2) was added to a stable therapeutic regimen. Clinical evaluation of disease activity and treatment side effects was performed before each infliximab infusion. Disease activity was evaluated according to DAS-28 index. Primary end-points were treatment safety profile and efficacy according to the EULAR response criteria. Peripheral blood monocytes (PBMCs) were collected from five patients at baseline and after 12 weeks, and were assessed for markers of activation (CD25 expression) by FACS analysis, with or without activation with PHA.

Results Sixteen patients completed 12 weeks of triple therapy. Three patients discontinued treatment because of adverse events: one for pneumonia, one for lymph node tuberculosis and one for gastrointestinal discomfort. Seven out of 18 patients (43.7%) achieved moderate response according to the EULAR response criteria. Statistically significant improvements in the mean values of tender joints (from 18.5 to 13.1; P = 0.04), swollen joints (from 18.6 to 12.1; P = 0.004), HAQ (from 1.2 to 0.7; P = 0.01), patients’ global assessment (from 67 to 46; P = 0.02) and pain (from 64 to 42; P = 0.04) were identified in those 16 patients. CD25 expression both in unstimulated and in PHA stimulated PBMCs was reduced (37 ± 34% to 15 ± 10%, and 50 ± 15% to 29 ± 20%, respectively).

Available online http://arthritis-research.com/supplements/6/S1
In this preliminary report, addition of CsA to a maximum
concentration of 0.11 between baseline and infliximab do not seem to increase EBV
load in patients with rheumatoid arthritis. Methotrexate and infliximab were studied. Of these 221 patients, 88 were
followed for 1–4 years: 19 received only methotrexate (eight were included in the study before introduction of methotrexate), eight received
only infliximab, and 81 received both methotrexate and infliximab. A 214 base-pair fragment from the EBV genome first internal repeat (IR1) was
amplified by quantitative real-time PCR to evaluate EBV DNA load.

Results EBV load decreased dramatically in most patients right after intro-
duction of methotrexate and kept decreasing thereafter. In patients receiv-
ing infliximab and methotrexate, three patterns of EBV load evolution were observed: stable, decreasing and variable. Patients with only infliximab had either stable or decreasing EBV loads. Although an increase in EBV load was observed in a few patients, none of them reached the
500 copies/500 ng level and no patient developed lymphoma.

Conclusions Methotrexate and infliximab do not seem to increase EBV load in patients with RA.

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Prediagnosis radiological progression in rheumatoid arthritis: a novel tool to study the effects of early DMARD therapy
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Objective The aim of the study was to determine whether a new method, based on intrapatient comparisons between estimated rates of
prediagnosis radiological progression and subsequent progression, can be used to assess the efficacy of commonly used disease-modifying
anti-rheumatic drugs (DMARDs) in a longitudinal early rheumatoid arthritis (RA) inception cohort.

Methods We analyzed clinical data and radiographs from 149 newly
diagnosed RA patients. Four groups were chosen: patients treated with methotrexate (MTX, n = 56), sulfasalazine (SSZ, n = 55) and auran-
in (AUR, n = 19), and a control group of patients who had changed therapy twice during the first 2 years after diagnosis and whom we pre-
viously presented as a group of poor responders with persistent clinical activity (control, n = 19). Radiographs were quantified by an experi-
enced reader using Larsen Erosion Score and the ‘X-RayRheuma-
Coach’ software. The rate of prediagnosis radiological progression per
time was estimated by taking the first onset of RA symptoms as the
starting date for radiographic damage.
Results The mean disease duration from onset of symptoms until diagnosis and DMARD institution was 6.7 ± 3.6 months. The mean baseline Larsen Score was 13.2 ± 9.3, resulting in an estimated prediagnosis progression rate of 23.6 ± 16.7 Larsen Score units/year. In the control group and in the patients receiving AUR, radiological progression after diagnosis was similar to the predicted progression based on the estimated prediagnosis progression rates. In patients for whom MTX or SSZ was the first-line therapy, a marked \( P<0.001 \) reduction in radiographic progression in the first year after treatment initiation was seen compared with prediagnosis progression.

Conclusions Prediagnosis rates of radiological progression can be used quantitatively to obtain important information on the potential efficacy of DMARDs, and indicate that MTX and SSZ, but not AUR, significantly retard radiographic damage in the first year after diagnosis.

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The effect of infliximab on skin lesions in a patient with scleroderma (CREST)
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Background Skin lesions refractory to treatment are a source of morbidity in patients with scleroderma (CREST) and may result in amputation. No established DMARD is available for this disorder.

Objective The aim was to report the effect of infliximab in combination with methotrexate (MTX) on refractory skin lesions in a 46-year-old woman with scleroderma (CREST).

Case report In 1991, the patient was diagnosed with scleroderma (CREST). Despite several treatments (initially D-penicillamine and colchicine, subsequently MTX, colchicine and misoprostol), she developed severe digital ulcerations, resulting in multiple amputations with difficult wound healing afterward. Since 1999, she was treated with MTX, colchicine and alprostadil. In August 2002 she developed necrosis at the fingertip of digit IV right. Despite hyperbaric oxygen treatment and pulse therapy with corticosteroids, the necrosis worsened. Therefore, infliximab (3 mg/kg IV at weeks 0, 2 and 6, and every 8 weeks thereafter) combined with MTX (10 mg/w im) was started. The patient also had ulcerations at digit II left and at the knees, and suffered from severe Raynaud’s attacks. After the second infusion, the ulcerations started to heal. Although an amputation of distal phalanx of digit IV could not be avoided, infliximab was continued. The amputation wound healed quickly, the ulcerations became considerably smaller, and she developed no new skin lesions. The inflammatory parameters remained stable and her general condition improved; she gained weight (6 kg), had less pain in the fingers and fewer Raynaud’s attacks, and her skin became more supple. Infliximab was stopped after eight infusions. Now, 4 months later, the previous ulcerations remain healed, no new digital lesions occurred and the inflammatory parameters are stable.

Conclusion We present a patient with scleroderma (CREST) and refractory skin lesions. Infliximab combined with MTX was well tolerated and effective in the healing of ulcerative lesions.

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Anti-TNF-α antibody Infliximab and glucocorticoids reduce serum vascular endothelial growth factor (VEGF) levels in patients with rheumatoid arthritis: a pilot study
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Background Pannus growth in rheumatoid arthritis (RA) is critically dependent upon accompanying neovascularization. Vascular endothelial growth factor (VEGF) is a potent angiogenic promoter, which is thought to play a crucial role in synovial angiogenesis in rheumatoid arthritis (RA). Serum VEGF levels are elevated in RA patients, and therapy with the anti-TNF-α antibody infliximab reduces serum VEGF levels in these patients.

Objective The study was conducted to compare the early effect of oral glucocorticoids (GC) with anti-TNF-α therapy (infliximab) on serum VEGF levels in patients with RA.

Methods Five RA patients (5/8) who had no prior treatment with DMARDs or glucocorticoids (GC) were administered 20 mg/d prednisolone. Three patients who failed more than one disease-modifying antirheumatic drug (DMARD) therapy received infusion with infliximab (200 mg). VEGF serum levels were measured using ELISA before treatment and at day 10 or 13 during prednisolone therapy or 14 days after the first infliximab infusion.

Results Serum VEGF levels in therapy naïve RA patients (GC group) were higher than those in pretreated patients who received infliximab (median serum VEGF level: 1106 pg/ml versus 320 pg/ml; \( P = 0.09 \)). Treatment with infliximab as well as glucocorticoids significantly decreased serum VEGF levels after 10–14 days in RA patients (median serum VEGF level after treatment: GC group 559 pg/ml, infliximab group 92 pg/ml; \( P = 0.01 \) versus without treatment or preinfusion).

Conclusions Treatment with oral glucocorticoids leads to an early and drastic reduction in serum VEGF levels in therapy naïve RA patients. TNF-α inhibition by infliximab could reduce low serum VEGF levels in pretreated RA patients independent of the initial serum VEGF concentration. This is the first report comparing the effect of both therapies on serum VEGF levels, indicating a possible therapeutic inhibition of angiogenesis in RA.

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Allogeneic bone marrow transplantation can suppress established and otherwise chronic collagen-induced arthritis after a nonmyeloablative conditioning regimen employing anti-CD40 ligand monoclonal antibody
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Objective Allogeneic bone marrow transplantation (BMT) may be more effective than autologous BMT as a treatment for patients with severe autoimmune diseases, such as rheumatoid arthritis (RA). However, the application of allogeneic BMT for treatment of RA is not yet feasible because of the high-dose immunosuppression that is used before and after BMT. Therefore, in this study collagen-induced arthritis (CIA) – the classic mouse model for RA – was used to compare the effects of allogeneic and syngeneic BMT after less toxic, nonlethal (i.e. nonmyeloablative) conditioning regimens on established disease in DBA/1 (H-2^d) mice.

Methods We induced arthritis in normal DBA/1 (H-2^d) mice by immunization with type II collagen (CII) in complete Freund’s adjuvants (CFA). To allow engraftment, we made use of anti-CD40 ligand monoclonal antibody (one injection of 0.5 mg ip) and nonlethal total body irradiation (TBI) of 6.0 Gy before BMT with 1.0 × 10^7 total BM cells from syngeneic DBA/1 (H-2^q) mice or from fully MHC-mismatched allogeneic BALB/c (H-2^d) mice. After treatment, mice were scored for clinical arthritis, and serum was taken to measure the amounts of CII-specific antibodies.

Results We were able to induce stable and long-term (>300 days) donor chimerism (>95%) after transplantation with fully MHC-mismatched total BM cells from allogeneic BALB/c (H-2^d) mice using a nonmyeloablative conditioning regimen. After initial exacerbation of arthritis shortly after allogeneic BMT, due to a graft-versus-host (GvH)/host-versus-graft (HvG) response, mice receiving allogeneic BM cells showed a significant suppression of arthritis and CII-specific anti-
These data indicate that nonmyeloablative conditioning to investigate a possible cross-reaction between Coxsackievirus RNA has recently been detected in synovial tissue (ST) from RA patients and appropriate controls between expression patterns and other parameters and to evaluate its expression on both levels in material derived from RA patients or healthy donors (HD).

**Methods** Synovial tissue (ST) from RA patients and appropriate controls was stained with a specific monoclonal antibody (immunohistochemistry). The expression of HDJ2 was characterized in peripheral blood (PB) mononuclear cells (MC) from HD; PBMCN from RA patients; and MNC from synovial fluid (SFMCN) and ST from RA patients. PB and SF leukocytes isolated by density gradient centrifugation were separated in monocytes (MC), remaining mononuclear cells (nonMC) and granulocytes. Cell populations were analyzed for HDJ2 expression by FACS and RT-PCR.

**Results** HDJ2 mRNA was expressed in PBMCN of HD. Separation of PBMCN in MC and nonMC showed HDJ2 mRNA in both populations. HDJ2 protein was detected intracellularly in PBMCN of HD by FACS analysis, with stronger signals in MC than in nonMC. PBMCN derived from HD and RA patients did not show any surface expression of HDJ2. PB and SF leukocytes from RA patients varied in their HDJ2 mRNA expression between presence and absence. HDJ2 protein was overexpressed in RA ST and detected on the cell surface of RA SFMCN. Conclusions HDJ2 mRNA and protein expression are regulated differentially. More patients must be analyzed to describe possible correlations between expression patterns and other parameters and to understand the function of synovial HDJ2 expression.

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**Effects of anti-rheumatic treatments on expression of microsomal prostaglandin E synthase-1 in rheumatoid arthritis synovium**

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**Background** mPGES-1 catalyzes the formation of PGE2 from COX-derived PGH2. mPGES-1 is induced by proinflammatory cytokines and linked to conditions with high PGE2 biosynthesis. Recent studies in animals suggest that mPGES-1 upregulation is involved in the pathogenesis of inflammatory arthritis, and thus has important clinical relevance for a better understanding and treatment of rheumatoid arthritis (RA).
Objective Our aim was to study the effects of different antirheumatic therapies on expression of mPGES-1 in RA synovial tissue.

Methods Synovial biopsies were taken by arthroscopy in patients with RA before and after TNF-blocking therapy with either etanercept (eight patients) or infliximab (10 patients), and in patients with chronic inflammatory arthritis before and after intra-articular injection of steroids (16 patients). Immunohistological analysis was performed using antibody against mPGES-1 and COX-2. Double immunofluorescence (DIF) was performed with antibodies to CD3, CD20, CD68, CD163 and prolyne-4-hydroxylase.

Results mPGES-1 staining was detected in synovial lining cells, in sublining scattered cells in all patients, and in endothelial cells in few patients. DIF showed that mPGES-1 is produced in synovial macrophages and fibroblasts, whereas in lymphocytes mPGES-1 expression was not observed. TNF-blocking therapy has heterogeneous effects on the expression of mPGES-1, as well as COX-2 in synovial tissues. Intrarticular steroid treatment significantly reduced both mPGES-1 and COX-2 expression in synovial tissues (P < 0.001); however, the downregulation of mPGES-1 appears to be more prominent than that of COX-2.

Conclusion The results demonstrate that corticosteroids but not TNF blockade downregulate mPGES-1. These data might be of importance for interpretation of effects of various therapies and provide support to the use of combination of TNF-blockade, corticosteroids, and nonsteroidal anti-inflammatory drugs.

Salivary gland epithelial cells (SGEC) express a novel alternate transcript of the B7.2 costimulatory molecule that lacks the CD28/CTLA4-binding IgV-like domain

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Background Epithelial cells appear to have a central role in the pathogenesis of Sjögren’s syndrome (SS), by acting as antigen-presenting cells. SGEC lines established from minor SG biopsies of SS patients express MHC and B7 costimulatory molecules. We previously showed that B7.2 molecules expressed by SGEC display distinctive binding properties denoted by the functional interaction with CD28 receptor and reduced binding to the negative immune regulator CTLA4.

Objective and Methods To delineate the role of RNA modifications in the SGEC–B7.2 protein binding properties, we applied a RT-PCR protocol that amplifies the entire coding region of the B7.2 mRNA. The amplified PCR products were sequenced and characterized.

Results SGEC were found to express three distinct alternate transcripts of the B7.2 molecule. These included the two previously described transcripts (encoding the full-length and the soluble form, respectively), as well as a third novel form (designated as B7.2C). B7.2C is characterized by exon 4 deletion, which encodes the IgV-like binding domain of the B7.2 protein to the CD28 and CTLA4 receptors. The conservation of the signaling peptide and the transmembrane region in the B7.2C transcript suggests the membranous expression of the encoded protein. The cell surface expression of the B7.2C protein was verified in CHO cell lines transfected with this transcript. B7.2C mRNA analysis of peripheral blood cell subpopulations (monocytes, T and B cells) revealed exclusive expression by monocytes.

Conclusion The role of B7.2C transcript remains to be determined. The clustering of B7.2 molecules and the development of a network has been considered necessary for their effective interaction with CD28/CTLA4 receptors. Thus, the co-expression of the full length B7.2 with the truncated B7.2C protein (that lacks the CD28/CTLA4-binding sites) may lead to negative regulation of T cell activation by interference of B7.2C in the formation of B7.2 clusters.

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Statins have no effect on collagen-induced arthritis in mice

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Background HMG-Co A reductase inhibitors (statins) are widely used lipid-lowering agents. It has recently been shown that, in addition to their well known effect on cholesterol levels, statins display anti-inflammatory activities both in vitro and in vivo. In this context, in vivo prophylactic and therapeutic effects of simvastatin were recently described in mouse collagen-induced arthritis, a well-described experimental model for human rheumatoid arthritis (RA).

Objective and Methods The aim of this study was to investigate further the in vivo effects of three different statins — atorvastatin, rosuvastatin and simvastatin — using the same experimental model. Different doses and routes of administration were used for the various statins in an attempt to elicit antiarthritic activity in preventive and curative treatment protocols.

Results Atorvastatin and rosuvastatin had no in vivo efficacy, as indicated by clinical, histological (synovial hyperplasia, exudate and cartilage damage), immunological (anti-type II collagen IgG production and T lymphocyte proliferation) and biochemical (IL-6, serum amyloid A and glucocorticoid production) parameters of inflammation and autoimmunity. The previously described antiarthritic effects of intraperitoneal simvastatin administration were reproduced in our experiments, but could be accounted for by very severe side effects of the treatment leading to increased glucocorticoid levels.

Conclusions This work shows that statins do not have any effect in a murine model of arthritis; this is an unexpected observation, given the previously described therapeutic effect of statins in immune-mediated inflammatory diseases. It is still unclear whether statins might have a benefit in rheumatic diseases.

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Serum MRP8/MRP14 as a biomarker for inflammation in autoimmune arthritis

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Background Myeloid related protein (MRP8) and MRP14 are released by early monocytes upon contact with activated endothelium and correlate with inflammation in rheumatoid arthritis (RA) and juvenile arthritis.

Objective The aim of the study was to investigate serum MRP8/MRP14 as biomarker for inflammation in chronic arthritis.

Methods The study included 40 RA patients with active joint involvement (SJC = 14, CRP = 2.4 mg/dl, ESR = 28 mm/hour); 30 SpA patients with peripheral synovitis (SJC = 2, CRP = 2.2 mg/dl, ESR = 21 mm/hour); 10 AS patients with exclusive axial involvement (SJC = 0, CRP = 3.1 mg/dl, ESR = 19 mm/hour); and 20 healthy controls (HC). In 20 RA, 10 peripheral SpA and 10 axial AS patients, sera were also obtained after 6 weeks of infliximab therapy. Serum MRP8/MRP14 was measured by ELISA.

Results MRP8/MRP14 was increased in RA (1075 ng/ml; P < 0.001), peripheral SpA (815 ng/ml; P < 0.001) and axial AS (710 ng/ml; P < 0.001), as compared with HC (280 ng/ml). In patients with peripheral synovitis, MRP8/MRP14 correlated with CRP (RA, r = 0.77, P < 0.001; peripheral SpA, r = 0.69, P < 0.001) and ESR (RA, r = 0.70, P < 0.001; peripheral SpA, r = 0.62, P = 0.001). MRP8/MRP14 decreased after infliximab in RA (P < 0.001), peripheral SpA
Power Doppler sonography is a reliable imaging modality for detecting new blood vessel formation (angiogenesis) in rheumatoid inflammatory processes. Vascularity near and inside the wrist of 23 RA patients and 12 healthy controls was visualized by power Doppler sonography. Microvascular Doppler flow was localized to either side or outside the joint capsule, and estimated according to a grading from 1 to 4. Serum levels of VEGF were measured using a standard quantitative sandwich ELISA.

**Results** Power Doppler mode identified increased synovial microvascular blood flow inside the joint capsule in 20 RA patients (87%), in comparison with none of the healthy controls (P < 0.0001). Although the median serum VEGF level in RA patients (853 pg/ml) was higher than in healthy controls (638 pg/ml), qualitative Doppler sonographic estimation of the intensity of intra-articular synovial blood flow did not correlate with the actual serum VEGF level of the same patient.

**Conclusion** Power Doppler sonography is a reliable imaging modality to detect increased intra-articular synovial microvascular blood flow as a result of vasodilatation and new blood vessel formation (angiogenesis) in acute arthritis of RA patients. A correlation with elevated serum VEGF levels indicates this method as a helpful tool to study the role of blood vessels in the rheumatoid inflammatory process.

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A new approach to study angiogenesis in rheumatoid arthritis by power Doppler sonography and serum vascular endothelial growth factor level measurement

**Objective** Because 3D power Doppler sonography plays an important role in obstetrics and oncology for assessing the vascular system, we used it to visualize intra-articular synovial new blood vessel formation in painful and swollen joints of patients with RA.

**Methods** Inflamed wrist, knee or finger joints in 21 patients with active RA were investigated by ultrasound. Vascularity near and inside the joint capsule was visualized by power Doppler function. The online 3D power Doppler function, provided by the ATL/HDI 5000 vascular software, was used to generate a 3D image of the periarticular and intra-articular blood vessels.

**Results** In 21 patients with RA who showed power Doppler signals in 3D, the 3D function revealed a blood vessel tree branching out from periarticular small blood vessels into the joint capsule. Until now it was possible to obtain good 3D imaging of synovial vasculature in the wrist (15 patients), knee (three patients), and finger joints (MCP II and PIP III) (three patients).

**Conclusions** This is the first report on 3D power Doppler imaging of synovial angiogenesis. In comparison with 2D power Doppler, 3D mode reveals more and very small blood vessels in connection with the whole blood vessel tree, and provides a new opportunity to study the architecture and morphological structure of synovial vasculature in various joint disorders and under different conditions. Thus 3D power Doppler sonography may be used as an *in vitro* model to study the mechanisms between inflammation and angiogenesis in rheumatoid joint damage.

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A new approach to study angiogenesis in rheumatoid arthritis by power Doppler sonography and serum vascular endothelial growth factor level measurement

**Objective** To develop a new approach to study angiogenesis in rheumatoid arthritis (RA) using 3D power Doppler sonography.

**Methods** Inflamed wrist, knee or finger joints in 21 patients with active RA were investigated by ultrasound. Vascularity near and inside the joint capsule was visualized by power Doppler function. The online 3D power Doppler function, provided by the ATL/HDI 5000 vascular software, was used to generate a 3D image of the periarticular and intra-articular blood vessels.

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**Conclusions** This is the first report on 3D power Doppler imaging of synovial angiogenesis. In comparison with 2D power Doppler, 3D mode reveals more and very small blood vessels in connection with the whole blood vessel tree, and provides a new opportunity to study the architecture and morphological structure of synovial vasculature in various joint disorders and under different conditions. Thus 3D power Doppler sonography may be used as an *in vitro* model to study the mechanisms between inflammation and angiogenesis in rheumatoid joint damage.
Objective To evaluate clinical and serological profile associated with anti-SL antibodies

Methods Twenty-six consecutive sera with anti-SL antibodies, selected from our laboratory routine ENA detection, were studied. All sera were analyzed by counter immunoelectrophoresis (CIE) for anti-ENA detection, using rabbit thymus and human spleen extract as substrates. ANA (by IFI assay on HEp-2 cells), anti-dsDNA (by Farr assay), anti-ENA, anticardiolipin (aCL) and anti-beta2GPI (by ELISA) were detected at disease onset and during follow up. The patients (20 female and 6 male) had a mean age at onset of 39.4 years (SD: 18.3 years) and a mean follow-up of 10.7 years (SD: 7.3 years).

Results All the patients were affected by autoimmune rheumatic diseases, but one with HCV-related cryoglobulinemia. SLE and primary Sjögren Syndrome (pSS) were the most frequent diagnoses achieved (in 13 and in 6 cases, respectively). Main clinical features were represented by skin involvement (65%), Raynaud’s phenomenon (38%), xerophthalmia (54%), xerostomia (34%) and serositis (15%). Nevertheless, severe manifestations of disease were frequently observed: CNS involvement in three (11.5%) and renal disease in four cases (15%), while one patient with SLE had primary pulmonary hypertension. No cases of hemolytic anemia or severe cytopenia were recorded. ANA positivity was observed in all the sera. Anti-SL antibodies were detected as isolated antibody specificity in only five patients, while nine sera showed reactivity to anti-SL plus one antinuclear specificity (5 anti-dsDNA, 2 anti-Ro, one anti-RNP, one anti-Sm). Ten patients had anti-SL antibodies associated with two other ANA specificities: in five cases, anti-Ro and anti-dsDNA; in three, anti-Ro and anti-La; in two cases, anti-Sm and anti-dsDNA. Two patients showed multiple antinuclear specificities (anti-SL with anti-Ro, anti-La and anti-dsDNA in one case and anti-SL with anti-Ro, anti-Ku and anti-dsDNA in another). Anti-cardiolipin and anti-beta2GPI were detected in seven and four patients, respectively.

Conclusion Anti-SL antibodies identify a group of patients mainly affected by SLE and pSS, presenting in approximately one third of patients a severe disease with pulmonary, CNS or kidney involvement. Twenty-one patients with anti-SL (81%) have multiple antinuclear specificities.

106 Drug induced differential gene expression in normal and RA synovial fibroblasts cell lines

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Background Rheumatoid arthritis (RA) synovial fibroblasts destructively invade into joint cartilage. We could demonstrate in our pannus model of interactive three-dimensional tissue culture that the immortalized synovial fibroblast cells from a normal donor (K4IM) and a patient with RA (HSE) provide an in vitro system for differential invasiveness into artificial cartilage.

Objective In this study, we investigated the molecular differences between these two cell lines and the influence of antirheumatic drugs on their expression profiles to gain insight into molecular pathways of joint destruction.

Methods HSE and K4IM cells were characterized by immunostaining with Fibroblast-AK, CD3, CD31, CD68 and MAC3 antibodies. MTS-assay provided information for kinetics and effective concentration of MTX, Decortin and diclofenac. Gene expression profiles were generated with HG-U133A Affymetrix GeneChips, analyzed by RMA, dCHIP and MAS5.0 software, and validated by RT-PCR, ELISA.

Results Positive fibroblast-AK staining confirmed the fibroblastoid phenotype of both cell lines. RA fibroblasts reacted more sensitively towards MTX-mediated cytotoxicity compared to K4IM fibroblasts. Gene expression profiling revealed 1303 differentially expressed genes between HSE and K4IM. MTX affected 191/1365, Decortin 84/172 and Diclofenac 19/0 genes in HSE/K4IM, respectively, as identified by all three softwares in common. Only 41, 26, or 8 genes were influenced in HSE towards the K4IM profile by MTX, Decortin or Diclofenac, respectively. Regulation of ten selected genes was confirmed by RT-PCR. Proteins in the supernatant from three of four genes revealed similar ratios by ELISA compared to transcription levels.

Conclusion Only a minority of differentially regulated genes in the RA fibroblasts was positively influenced by current therapeutics. Those genes, which were not influenced, may be new potential candidates for therapeutic intervention.