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Aetiopathogenesis of rheumatic diseases

1

HIF-1 mediated upregulation of VEGF and VEGF-R in systemic sclerosis (SSc): Imbalance with angiostatic factors suggests VEGF as a novel option for the treatment of ischemia in patients with SSc

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Vascular changes are consistent early findings in patients with SSc and often precede the development of fibrosis. Despite a significant reduction in the capillary density, there is paradoxically no sufficient angiogenesis in the skin of SSc patients. By using a pO_2 histograph, we showed that low pO_2 values are overt in involved skin of patients with SSc. *In vitro*, real-time PCR revealed a 3.7-fold upregulation of the potent angiogenic growth factor VEGF in SSc fibroblasts after hypoxic exposure compared to normoxic controls. *In situ* hybridization for VEGF in skin biopsies of patients with SSc showed an overexpression of VEGF mRNA by fibroblasts and mononuclear infiltrates, whereas its expression was limited to keratinocytes in healthy control biopsies. In contrast to the SSc skin, HIF-1 alpha protein was found to be coexpressed with VEGF in healthy skin samples, indicating that the constitutive VEGF synthesis in the skin is driven by this transcription factor. Additionally, we showed that the lack of angiogenesis in SSc is not due to a reduced bioavailability of the overexpressed VEGF, since the VEGF receptors Flk-1 and Flt-1 were found to be expressed on endothelial cells of patients with SSc, but not in healthy controls, and since SSc patients had severely elevated serum levels of VEGF compared to healthy controls. Despite the enhanced levels of VEGF, serum samples of SSc patients did not induce angiogenesis in the vivo chorion allantois membrane assay, indicating that the proangiogenic effects of VEGF may be outweighed by angiostatic factors. The hypothesis that VEGF synthesis has to be above an individual threshold in SSc patients to induce angiogenesis was further strengthened by the finding that patients without fingertip ulcers had significantly higher levels than patients with fingertip ulcers. Interestingly, the angiostatic factor endostatin was elevated in a subset of patients and thus may counteract directly the biologic effects of VEGF in SSc patients. Serum levels of VEGF were also correlated significantly with disease severity parameters including antitopoisomerase antibodies. These results suggest that therapeutic application of VEGF by either gene transfer or as a recombinant protein might be a novel option in SSc.

2

Comparison of the features of arthroscopic synovial biopsies with biopsy samples obtained at surgery

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Objective: Most of the older descriptions of the synovial infiltrate are based on examination of synovial tissue (ST) obtained at surgery. However, ST from end-stage destructive rheumatoid arthritis (RA) and arthroscopic biopsies obtained during active inflammation could exhibit different characteristics. The aim of this study was to define the cell infiltrate, the expression of proinflammatory cytokines, angiogenic factors, and matrix metalloproteinases in ST selected at arthroscopy compared with ST from end-stage RA obtained at joint replacement.
Methods: Synovial biopsy specimens were obtained from the actively inflamed knee joints of 11 RA patients with longstanding RA by arthroscopy and compared with ST from 13 patients with end stage, destructive RA requiring joint surgery. Use of medication was on average similar in the 2 groups. Immunohistologic analysis was performed using monoclonal antibodies (mAb) to detect T cells, plasma cells, macrophages, fibroblast-like synoviocytes (FLS), as well as the expression of IL-1β, IL-6, and TNF-α, matrix metalloproteinase (MMP)-1, MMP-3, MMP-13, tissue inhibitor of matrixmetalloproteinase (TIMP)-1, and vascular endothelial growth factor (VEGF). The integrated optical density was evaluated by computer-assisted image analysis.
Results: The expression of CD68+ macrophages was significantly higher in ST selected at arthroscopy compared to samples obtained at surgery, both in the intimal lining layer and in the sublining layer. The expression of CD3+ T cells also tended to be higher in arthroscopic samples. There was no clear difference in the expression of $CD38^+$ plasma cells and $CD55^+$ FLS. The expression for TNF-α, IL-6, MMP-1, MMP-3, MMP-13, TIMP-1, and VEGF was on average higher in ST obtained at arthroscopy. The expression of IL-1β was on average higher in ST obtained at surgery.

Conclusion: Active arthritis activity is especially associated with increased cell infiltration, expression of proinflammatory cytokines, MMPs, and angiogenic growth factors in synovial biopsy samples selected at arthroscopy. Increased expression of IL-1β in the synovium of patients with destructive RA requiring joint replacement may well reflect the important role of IL-1β in cartilage and bone destruction.

3

Epstein-Barr virus load in rheumatoid arthritis patients and normal controls: accurate quantification using real time PCR

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Objective: For twenty years the Epstein-Barr Virus (EBV) has been suspected to contribute to the pathogenesis of rheumatoid arthritis (RA). RA is strongly associated with shared epitope positive HLA-DR alleles. EBV load has been extensively studied in RA patients, using semi-quantitative PCR. Inconsistent results reflect the lack of sensitivity and accuracy of this technique. We quantified EBV in peripheral blood mononuclear cells by real time PCR, to (1) determine whether EBV load is higher in RA patients compared to controls and (2) test whether HLA-DR alleles influence EBV load in RA patients and controls.
Methods: Fifty patients fulfulling the 1987 ACR criteria for RA were studied. Most patients were treated with DMARDs including methotrexate, leflunomide, etanercept or infliximab. Fifty healthy controls were chosen from bone marrow donors at the Marseille blood transfusion center. HLA-DR genotyping of patients and controls was performed by PCR-SSP. Real time PCR was performed using a Roche LightCycler. A 214 bp fragment from the highly conserved long Internal Repeat IR1 was amplified. IR1 is repeated eleven times in the EBV genome, increasing the sensitivity of detection. Two specific hybridization probes were used to recognize adjacent internal sequences within the target. EBV-positive Burkitt's lymphoma cell line was used as an external standard.
Results: EBV load is expressed in EBV genome copy number per microgramm of human genomic DNA. Preliminary results show a higher EBV load in RA patients (0–60 copies/μg) than in normal controls (0–10 copies/μg). We are currently testing the influence of HLA-DR genotypes on EBV load in controls.

4

Low levels of apoptosis and high FLIP expression in early rheumatoid arthritis synovium

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Objectives: To define synovial apoptosis with respect to disease duration, inflammatory cell type, FLIP (FLICE like inhibitory protein) and cytokines expression in patients with rheumatoid arthritis (RA).
Methods: Synovial biopsy specimens from eleven patients with longstanding RA (median disease duration 21 years) and eight with early RA (median disease duration 5 months) have been investigated. We evaluated apoptosis (TUNEL method combined with morphologic analysis), cell surface markers (CD3, CD68), cytokines (IL-1α, IL-1β, TNF-α and IL-6) and FLIP expression. Computer-assisted image analysis was used for quantification.
Results: Apoptosis level in RA synovium was significantly higher in the group of patients with long standing RA than in the patients with early RA (8.8% versus 0.6%, $P = 0.001$), while number of macrophages and FLIP expression were higher in the early as compared with long standing RA group (16.2% versus 8.3%, $P = 0.02$ and 31.1% versus 0.2%, $P = 0.001$ respectively). All three markers significantly correlate with disease duration ($r = -0.7$, $P < 0.001$ for FLIP, $r = 0.6$, $P = 0.001$ for apoptosis and $r = -0.5$, $P < 0.05$ for CD68). Cytokine expression and T cell scores were not significantly different in early RA compared to longstanding RA. We did not observe differences between corticosteroids treated versus corticosteroids non-treated patients or between DMARD treated versus non-treated patients.
Conclusions: Our findings suggest that RA synovial macrophages are resistant to apoptosis in early RA and express high levels of FLIP. During natural or drug modified disease progression the apoptotic mechanism may be restored with a specific increase of synovial apoptosis in patients with long standing arthritis.

5

Intestinal anaerobic bacteria in early rheumatoid arthritis (RA)

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Increasing attention has recently been paid to the normal intestinal flora as a potential source of etiological agents in RA. Changes in the intestinal flora, due to fasting or diet, have been shown to reflect improvement of the patients when they are divided into high- and low-responders. Previously, evidence has also been presented that intestinal flora in the early RA is different from that of non-RA controls, due primarily to anaerobic bacteria.
The present study was designed to compare the fecal microbiota of the patients with early RA with the microbiota of the control patients using 16S rRNA oligonucleotide probes, detecting a variety of anaerobic bacteria in the normal intestinal flora. Fecal samples of 25 early, disease modifying antirheumatic drugs, naive RA patients and 23 control patients suffering from noninflammatory pain were investigated. The contribution of five bacterial groups was determined by using whole cell hybridization with seven fluorescently labeled 16S rRNA-targeted oligonucleotide probes. These probes cover one third to a half of the total bacteria in the human intestine. Patients with early RA had significantly less bacteria belonging to the Bacteroides, Prevotella and Porphyromonas genera than the controls (4.7% vs. 9.5%, $P = 0.00005$). The finding was confirmed with a probe specific for bacteria of the Bacteroides fragilis group (1.6% vs. 2.6%, $P = 0.02$). The samples of RA patients and the controls did not differ significantly when five other oligonucleotide probes were applied. They were detecting bacteria in the genera Atobium, Coriobacterium, Collinsella, Bifidobacterium and Fusobacterium, and in the Eubacterium rectale-Clostridium coccoides group. We conclude that the content of anaerobic bacteria in the intestinal flora of the patients with early RA is significantly different than that of the controls. The number of bacteria belonging to the Bacteroides-Prevotella-Porphyromonas group was, on average, in RA patients only half that of the controls. If this finding can be confirmed, together with a recent suggestion that certain Bacteroides species are required for fortification of the barrier function in the intestinal epithelium, it adds further evidence to the hypothesis that intestinal bacterial flora plays a role in the etiopathogenesis of RA.

6

Prevalence of antibodies against a Sindbis-related (Pogosta) virus, a potential cause of chronic arthritis

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A disease characterised by arthritis, rash and fever was described in Northern Finland in 1974 and named, according to the region, Pogosta disease. It closely resembles Ockelbo disease in Sweden, and Karelian fever, occurring in Western Russia. When analysing the clinical picture during an outbreak we found that 93% of the patients had joint inflammation, 40% with polyarthritis. Rash was seen in 88% of the patients, and 23% had fever. It has been suggested that the disease is self-limiting, but in a follow-up study we found that 50% of the patients suffered from chronic muscle and joint pain at least 2.5 years after the initial symptoms. There have been several outbreaks of Pogosta disease in Northern Karelia. They seem to occur every seven years. It has been assumed that Pogosta disease is locally restricted, as is described also for Ockelbo disease and Karelian fever. All three diseases are attributed to Sindbis-related arboviruses, and the spreading vector appears to be the late summer mosquitoes. Pogosta disease is considered to affect mostly young adults and middle-aged people. In an epidemiological study we analysed, using a semi-purified Sindbis-virus as antigen, antibodies against Pogosta disease in 2250 serum samples. Four hundred sera were from healthy blood donors and 1850 samples from patients who were suspected to have some viral infection. The samples represented different parts of Finland. Eleven percent were positive for IgG and 0.6% for IgM class antibodies. The antibody prevalence was almost equally distributed throughout the country, highest in Western Finland (17%). Of all samples with IgG class antibodies 25% were taken from children below 10 years.

Three conclusions can be made: 1) Pogosta disease is more common than was thought until now. 2) It is not only restricted to Eastern Finland but is spread throughout the whole of Finland. 3) It is also common in children, in contrast to an earlier belief.

7

Production of IFN-α by Natural IFN-α Producing Cells (NIPC), induced by apoptotic cells and autoantibodies via FcγRII, could be a pivotal event in the etiopathogenesis of SLE

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Background: Patients with SLE have an activated type I IFN system, and serum IFN-α levels correlate to both disease activity and severity. We have shown that SLE patients have an IFN-α inducing factor (SLE-IIF) in serum, consisting of anti-DNA antibodies and DNA in complex. The DNA could originate from apoptotic cells that are present in increased numbers in SLE patients. We recently demonstrated that IgG from SLE patients, but not normal individuals, together with apoptotic cells stimulate NIPC to produce IFN-α. In the present study we further characterized the interferogenic cell material and the role of different FcR on NIPC for the IFN-α response.

Methods: Apoptosis was induced in U937 cells by treatment with UV light and cell supernatant was collected at different time points. Normal PBMCs, costimulated with IFN-α, were cultured with the apoptotic cell material together with purified IgG from SLE patients or from normal individuals, and produced IFN-α was measured in culture supernatants. In some experiments the SLE-IgG was treated by papain or pepsin to obtain Fab and F(ab′)2 fragments, respectively. The effect on the IFN-α response by antibodies to CD16, CD32 (FcγRII), and CD64 as well as aggregated IgG was also investigated.

Results: Apoptotic cells release IFN-α inducing material in a time-dependent fashion and more than 1000 U IFN-α/ml was produced in the PBMC cultures, but only when the apoptotic cell material was combined with intact SLE-IgG. Normal IgG, SLE-IgG Fab or F(ab′)2 fragments together with apoptotic cell material where unable to induce IFN-α production. Heat-aggregated IgG and anti-CD32 antibodies inhibited the IFN-α response, whereas antibodies to CD16 and CD64 had no effect on the IFN-α response.

Conclusion: NIPC are induced to IFN-α production via the FcγRII by SLE autoantibodies and apoptotic cell material. This observation may explain the observed ongoing IFN-α production in SLE patients and may be of importance for the understanding of the pathogenesis of SLE.

8

Serum amyloid P component (SAP) binds to late apoptotic cells and mediates their phagocytosis by macrophages

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Background: Serum components, like serum amyloid P component (SAP), together with membrane receptors on phagocytes play essential roles in the phagocytosis of apoptotic cells. Disturbances in one of these factors might reduce phagocytosis and induce autoimmunity. SAP binds to apoptotic cells. SAP deficient mice spontaneously develop autoimmunity. We evaluated SAP binding to early and late apoptotic cells and whether this binding has functional consequences for the phagocytosis of these cells.

Methods: Human peripheral blood monocytes were isolated and cultured for 7 days to obtain monocyte derived macrophages. Jurkat cells were irradiated with UVB to induce apoptosis. After 4 hours 40% of cells stained with annexin V, and were propidiumiodide negative (early apoptotic cells, EA). After 24 hours 65% of cells were annexin V and propidiumiodide positive (late apoptotic cells, LA). EA and LA cells were incubated with FITC labeled SAP in the presence or absence of Ca^{2+} and subsequent binding was measured by flowcytometry. Phagocytosis was performed by incubation of macrophages for 30 minutes with EA or LA cells in the presence of human serum (HS) and depicted as phagocytosis index (PI, number of Jurkatt internalized by 100 macrophages). Experiments were repeated with SAP depleted serum and after reconstitution with different concentrations of SAP.

Results: Sixty percent of LA cells did bind SAP in the presence of Ca^{2+}, whereas the EA cells did not. SAP depletion of serum resulted in a 50% decrease of PI for LA cells, and complete restoration of PI could be demonstrated with SAP reconstitution up to 100 μg/ml. SAP depletion had no effect on PI of EA cells.

Conclusion: SAP binds to late apoptotic cells and is involved in the phagocytosis of these cells by human monocyte derived macrophages. This might have consequences for diseases in which phagocytosis of early apoptotic cells is decreased.

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Expression of syndecan-1 during development, growth and cartilage degeneration in a transgenic mouse model for osteoarthritis

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Mice heterozygous for the Del1 transgene locus with short deletion mutation in type II collagen gene develop degenerative changes in the knee joints from the age of 3 months which progresses to an end-stage OA by the age of 12–15 months. This study focuses on expression and distribution in syndecan-1 during development of osteoarthritic cartilage degeneration. Human samples from cartilage of osteoarthritic patients was studied for comparison. Northern analysis of total RNA extracted from knee joints of transgenic Del1 mice and their nontransgenic controls was used to monitor changes in syndecan-1 levels during development, growth, aging and cartilage degeneration. Immunohistochemistry was used to study the distribution of syndecan-1 in mouse and human samples. Syndecan-1 was present in the knee joints during development, growth and aging both in the control and Del1 mice, the mRNA levels being highest in the aged and late osteoarthritic samples. The most intensive immunostaining of syndecan-1 was seen in synovial tissue and adjacent of the defected areas of cartilage and menisci. In addition, some individual cells or cell clusters in the superficial zone of articular cartilage contained syndecan-1. In human osteoarthritic cartilage, dedifferentiated syndecan-1 positive cells were seen in corresponding locations to those in Del1 mice. We demonstrated syndecan-1 for the first time in aging and degenerating murine articular cartilage and synovial tissue. Syndecan-1 is involved in phenotypic modulation of the articular chondrocytes and during osteophyte formation. In this Del1 mouse model, proliferation plays a role forming characteristic chondrocyte clusters near the surface, while apoptosis occurs primarily in the calcified cartilage. These results suggest that syndecan-1 has a role in the functional activity of the chondrocytes during the disease process. Control of syndecan expression in articular cartilage could be an attractive target for therapeutic interventions in the future.

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Osteoclasts are essential for TNF-mediated joint destruction

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Recent studies suggest that osteoclasts may contribute to bone erosions in the joints of animal models of arthritis and human rheumatoid arthritis. We therefore adressed the question, can bone destruction occur in an osteoclast free model of arthritis? To answer this question, c-Fos knockout mice (c-fos–/–) were crossed with mice overexpressing human soluble TNF (huTNFtg). C-fos–/– mice lack osteoclasts and are therefore osteopetrotic since c-fos is essential for the signaling of osteoclast differentiation. HuTNFtg mice develop a severe and destructive arthritis through the signaling of huTNF via the p55 TNF receptor. The resulting four groups of mice (wildtype, huTNFtg, c-fos–/– and c-fos–/–/huTNFtg) were followed over 10 weeks and assessed for joint inflammation and joint destruction. Clinical features of arthritis, such as paw swelling and reduction in grip strength progressed equally in both huTNFtg and c-fos–/–/huTNFtg mice. Clinical features of arthritis were absent in c-fos–/– and wildtype mice. Quantitative histological evaluation of joint sections revealed no difference between huTNFtg and c-fos–/–/huTNFtg mice in the size of inflammatory synovial lesions. As previously described, huTNFtg mice showed severe bone erosions in all joint compartments. Bone resorption was characterized by the abundant presence of osteoclasts, as confirmed by cells positive staining for TRAP and the calcitonin receptor. Furthermore, the number of osteoclasts and the size of bone erosions were significant. In contrast, c-fos–/–/huTNFtg mice did not show any form of bone destruction despite the presence of severe inflammatory changes. C-fos–/–/huTNFtg mice were confirmed to lack osteoclasts by negative TRAP staining and the presence of osteopetrosis. Controls (c-fos–/– mice and wildtype mice) did not show histological signs of inflammation or bone erosion. In conclusion, these data clearly show that TNF-mediated bone erosion is triggered by osteoclasts, and the absence of osteoclasts turns TNF-mediated arthritis from destructive to non-destructive arthritis.

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Interaction of intimal fibroblasts with intracavitary fibrin: a morphologic follow up in ovalbumin arthritis

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Background: An imbalance between haemostasia and fibrinolysis, and subsequent fibrin generation within the rheumatoid joint could have a role in disease perpetuation.
Objective: To study fibrin formation at the synovial space in a model of rheumatoid arthritis, and its possible role in activating the synovial cells from inside of the cavity.
Methods: Antigen arthritis was induced by injecting ovalbumin into rabbits' knees. We looked for the appearance of fibrin in the effusion and at the inflamed tisues with immunohistochemistry, in a sequential fashion (from 24 hour to 1 week after disease induction). Morphologic changes at the intimal synovial surface in contact with fibrin matrices were studied over a long period of time by several qualitative variables. Analysis of the variables was carried out with Kruskall Wallis and Mann Whitney nonparametric tests, and linear regression was performed using the least squares method.
Results: Fibrin aggregates appeared from the initial stages of the disease at the synovial effusion. Later on, they were localised on the synovial surface. Differentiation of the aggregates from the underlying synovial tissue was easy at the beginning, but then progressive changes were noted at the fibrin-tissue interface, ending with the invasion of the aggregates by synovial cells and their incorporation into the tissue. The process involved cross-linking of fibrin matrices with fibronectin, and synoviocyte proliferation and migration.
Conclusion: Fibrin aggregates generated inside the joint cavity may constitute a source of activation and acquisition of invasiveness of synovial fibroblasts, a process to explore between the perpetuating mechanisms of rheumatoid arthritis.

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Vascular endothelial growth factor (VEGF) expression in muscle tissue and the effect of corticosteroid therapy in patients with poly- and dermatomyositis

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Background: Previous studies on pathogenic mechanisms indicate that the microvessels have a role in the disease mechanisms in polymyositis (PM) and dermatomyositis (DM). A reduced number of capillaries has been reported and this observation together with the increased expression of interleukin-1 and TGF-β suggest that there might be an hypoxic condition in the inflamed muscle tissue that could explain some of the clinical symptoms.
Aim of study: To further test this hypothesis we investigated if vascular endothelial growth factor (VEGF), which is upregulated by hypoxia, is expressed in muscle tissue in patients with PM and DM. A second aim was to investigate whether VEGF expression is affected by corticosteroid therapy.
Patients and methods: Six patients with PM and 4 with DM were investigated. A first muscle biopsy was taken at diagnosis and a second after 3–6 months with corticosteroid therapy. VEGF expression was investigated by immunohistochemistry using a rabbit polyclonal IgG antibody. Both conventional microscopic evaluation and computerised image analysis were used for evaluation of VEGF expression.
Results: These are our preliminary data: First biopsy: with conventional microscopic evaluation VEGF expression was observed in the endothelial cells of the microvessels in 9/10 patients and in larger vessels such as arterioles and venules in all patients. VEGF was also expressed in muscle fibres in all, and in mononuclear inflammatory cells in 3/10 patients. In the second biopsy, VEGF expression was still present in endothelial cells of capillaries and larger vessels as well as in muscle fibres, but with a seemingly weaker expression in the endothelial cells of PM patients and an increased expression in the DM patients. With computerised image analysis the results were similar.
Conclusion: VEGF is expressed in endothelial cells of capillaries and slightly larger vessels, in muscle fibres, and in occasional inflammatory cells in muscle tissue from patients with poly- and dermatomyositis. After corticosteroid therapy the expression decreased in some patients and increased in other patients. Whether or not the changed VEGF expression has any clinical significance and correlates with changes in muscle function still needs to be analysed.

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Etiology of a spontaneous autoimmune joint disease in mice

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Aims: To reveal which microbes are capable of inducing Ankylosing Enthesopathy (ANKENT), a spontaneous joint disease in susceptible mouse strains. Besides gender and age (young males afflicted) and genes (B57B6 background with some H-2 haplotypes being more effective), environmental factors (stress and microflora) have also been suggested to play a role in ANKENT onset. We have recently shown that ANKENT does not develop under germfree (GF) conditions.
Materials and methods: To identify ANKENT-triggering bacteria we transferred B10.BR ANKENT-prone mice into germfree conditions. Individual colonies were then associated with selected microbial cocktails. The incidence of ANKENT and immune system development has been studied in these gnotobiotic colonies.
Results: When compared to GF and conventional (CV) males (prevalence of 0 and ~20%, respectively), high incidence (~20%) of ANKENT has been revealed in mice associated with a cocktail of bacteria isolated from the intestine of an ANKENT-afflicted CV male. In the cocktail, no strong pathogens and Enterobacteriae like E. coli or Salmonella were present. The first ANKENT case has also been observed in mice colonized with a more restricted cocktail containing two selected gram-positive microbial strains. Surface phenotype of lymphocytes isolated from systemic lymphatic tissues, MALT and diseased joint were characterized. No significant differences in lymphatic tissues were detected between individual experimental groups. However, the prevalence of $CD4^+$ cells among joint-infiltrating lymphocytes was recorded.
Discussion: The presence of viruses, eukaryotic micro-organisms and noncultivable bacterial species (like segmented filamentous bacteria) is not required for ANKENT incidence. Although the autoimmune nature of the disease has not definitely been proven yet, the existence of specific ANKENT-triggering microbes strongly supports this hypothesis. The ANKENT-triggering agents are currently characterized by rRNA analysis.

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Investigation of serum cartilage oligomer protein (COMP) levels in rheumatoid arthritis (RA)

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RA is a disease characterised by an inflammatory process in the synovium and a degradation process of the joint cartilage. When articular cartilage matrix is degraded by a disease process, protein fragments are produced and some of them subsequently appear in the blood circulation and can be used to monitor cartilage degradation. COMP was first described by D. Heinegard as a noncollagenous protein primarily found in articular cartilage. COMP levels of serum and synovial fluids have been shown to have potential as prognostic markers of osteoarthritis and RA progression as well.
For the detection of COMP mainly monoclonal antibody based competition ELISA systems have been described. Recently on the basis of the research group of D.Heinegard a two-site sandwich ELISA test from AnaMar Medical AB has been available in wich two monoclonal antibodies directed against separate antigenic determinant of COMP molecule are applied.
Using the COMP ELISA test of 'AnaMar' we measured the serum COMP levels of 47 patients who fulfilled the ACR criteria of RA. Duration of the disease varied between 3–6 years. CRP and RF levels were also measured from the same sample. Patients were grouped by clinical activity, radiological progression and also by RF and CRP positivity. Our results showed that there was no difference between the serum COMP levels of seropositive and seronegative patients (11.4 vs. 10.71 ng/ml). Serum COMP levels of clinically active patients (11.7 ng/ml) were higher than of patients with inactive disease (10.5 ng/ml) ($P<0.08$). The average COMP levels of the radiologically progressive group was higher (12.5 ng/ml) than that of the non-progressive ones (10.3) ($P<0.06$). Statistically the highest difference was found between patients with elevated CRP (12.3 ng/ml) and those with normal CRP levels (8.8 ng/ml) ($P<0.03$). Significant correlation was also found between serum CRP and COMP levels R:0.54 ($P<0.003$). These results confirm previous findings that the serum COMP level reflects the actual degree of cartilage destruction ongoing during the inflammatory process of arthritis, thus can be a useful marker in predicting radiological progression and in monitoring the effectiveness of the treatment of RA.

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Increased FcγRII and III expression in synovium and on monocyte derived macrophages of RA-patients results in altered function after immune complex stimulation

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Introduction: Rheumatoid arthritis (RA) is characterized by extensive deposition of immune complexes (ICs) in the synovium. These ICs can communicate with resident macrophages and inflammatory cells entering from the circulation using FcγReceptors (FcγRs).
Objective: To determine whether macrophages of RA patients express different levels of FcγRs and whether this difference results in altered production of inflammatory mediators after stimulation with immune complexes.
Methods: Monocytes were isolated from blood of 10 RA-patients and 10 healthy controls and cultured for 7 days with M-CSF to obtain macrophages. Using FACS analysis, the expression of FcγRI, II and III was determined. At day 7 cells were stimulated with heat aggregated gamma globulins (HAGG) and 24 hours thereafter cytokine production was measured. In addition, immunohistochemistry was performed on synovial biopsies of knee joints of 27 RA patients and 5 controls. FcγRI, II and III were detected, as well as several inflammatory mediators.
Results: Macrophages derived from PBMC of RA patients showed a significantly higher expression of FcγRII (45%) and FcγRIII (15%) compared to controls. When RA cells were stimulated with HAGG, we found higher TNFα production. Also, when matrix degrading gelatinase/collagenase was detected, a significantly higher activity of these enzymes was found in the supernatants of HAGG stimulated RA macrophages vs. controls. Underlining these findings, we found highly significant positive correlations between the expression of FcγRII and III and the degree of inflammation in the joint in RA patients, but not for FcγRI. FcγRII and III expression was higher (respectively 80% and 125%) in RA synovium compared to controls. TNFα expression in the synovium was correlated with FcγRIII expression (r=0.51). MMP-1 expression was strongly correlated with FcγRI, II and III (respectively r=0.48, 0.60 and 0.62). FcγR expressions also correlated well with other cytokines, for example, IL-18 (positively: r=0.63) and IL-12 (negatively: r=−0.46).
Conclusion: Macrophages of RA patients express higher levels of FcγRII and III, resulting in elevated production of TNFα, and MMP-1. In addition, differences in FcγR expression in the synovium may also lead to different cytokine patterns. These data suggest that disturbed FcγR expression plays a role in RA pathology.

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IL-18 expression in synovial biopsies of patients with active rheumatoid arthritis is associated with enhanced levels of both IL-1 and TNFα

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Objective: The present study was performed to examine the expression patterns of IL-18 in synovial biopsies of patients with active RA. In addition, we determined whether expression of this primary cytokine was related to expression of TNFα, IL-1β, IL-12, IL-17, and adhesion molecules or cell markers.
Methods: Synovial knee biopsies were taken from 29 patients with active RA and were immunohistochemically stained for TNFα, IL-1β, IL-12, IL-17, and IL-18. Furthermore, ICAM-1, VCAM-1, E-selectin, CD3, CD14, and CD68 were stained. Both paraffin and cryo-sections were used for the detection of cytokines, adhesion molecules or cell markers. Five biopsies per patient were analyzed.
Results: IL-18 staining was detectable in 80% of the RA patients both in lining and sublining. TNFα was present in 50% of the RA-patients, whereas IL-1β was seen in 90% of the patients. When staining for TNFα was positive, variable location of TNFα was seen in the synovial lining, sublining layer and endothelial cells. IL-1β staining was consistent in all three compartments. IL-12 was predominantly expressed in the sublining in 59% of the RA patients, whereas only 24% of the patients stained positive for IL-12 in the lining. Of interest, IL-17 staining was obvious in 70% of the RA patients, and only seen in the sublining layer. ICAM-1 and E-selectin staining was only seen in the endothelial cells, whereas VCAM-1 was noted in the synovial lining and endothelial cells. IL-18 expression in the synovial lining was positively correlated with both IL-1 ($r=0.71$, $P<0.0001$) and TNFα ($r=0.68$, $P<0.0008$). In addition, IL-18 expression correlated with both microscopic inflammation scores ($r=0.78$, $P<0.0001$) and macrophage marker CD68 ($r=0.64$, $P<0.0007$) expression. Furthermore, IL-18 was positively correlated with both acute phase markers ESR ($r=0.61$, $P<0.0004$) and CRP ($r=0.57$, $P>0.001$).
Conclusion: Our results showed that IL-18 expression is associated with elevated levels of TNFα, IL-1β in synovial biopsies of patients with active RA. In addition, synovial IL-18 expression correlates with both acute phase markers ESR and CRP. These data indicated that IL-18 is a primary proinflammatory cytokine in RA that drives local IL-1/TNFα production and may be involved in enhanced acute phase protein levels.

Gene regulation and genetics

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A gene in the telomeric HLA complex distinct from HLA-A is involved in predisposition to juvenile idiopathic arthritis (JIA)

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Objective: Juvenile idiopathic arthritis (JIA) is associated with particular alleles at three different Human Leukocyte Antigen (HLA) loci: HLA-A, -DR/DQ and -DP. These associations are independent of each other; i.e. they cannot be explained by the known linkage disequilibrium (LD) between alleles at these loci. The purpose of this study was to look for additional JIA susceptibility genes in the HLA complex.
Methods: We investigated 102 Norwegian JIA patients and 270 healthy individuals, all carrying the DQ4-DR8 haplotype, by scanning ~10 Mb of DNA covering the HLA complex for microsatellite polymorphisms. An expectation-maximization (EM) algorithm was used to estimate haplotype frequencies, and the distribution of microsatellite alleles on the high-risk DQ4-DR8 haplotype was compared between patients and controls, to exclude effects secondary to LD with these susceptibility genes.

Results: Allele 5 at the microsatellite locus D6S265 (D6S265*5), 100 kb centromeric of HLA-A, showed strong positive association with disease (OR=4.7, Pc<10–6). Haplotype analysis demonstrated that the D6S265*5 association was not caused by LD to the gene encoding HLA-A*02, which has previously been described also to be associated with JIA. Rather our data suggest that a gene in LD with D6S265*5, but distinct from HLA-A*02, is involved in predisposition to JIA.
Conclusion: We found that D6S265*5 could be a marker for an additional susceptibility gene in JIA which is distinct from A*02, adding to the risk provided by DQ4-DR8.

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Differential gene expression of proliferating synovial fibroblasts in rheumatoid arthritis

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Objective: The aim of this study was to investigate the expression profile of rheumatoid arthritis synovial fibroblasts (RA-SF) during proliferation, and to explore the molecular mechanisms of synovial proliferation in RA.
Methods: Total RNA was extracted from 2 cultures of RA-SF, low-density (LD) proliferating cells and high-density (HD) nonproliferating cells, respectively, and suppression subtractive hybridization (SSH) was performed to compare differential gene expression of these 2 cultures. Subtracted cDNA was subcloned, and nucleotide sequences were analyzed to identify each clone. Differential expression of distinct clones was confirmed by semiquantitative RT-PCR. The expression and distribution of novel genes in synovial tissues was examined by *in situ* hybridization.
Results: Forty-four clones were upregulated in LD cells, and 44 clones were upregulated in HD cells. Forty-six of the 88 clones were identical to sequences that have previously been characterized. Twenty-nine clones were identical to cDNAs that have been identified, but with unknown functions so far, and 13 clones did not show any significant homology to sequences in the GenBank (NCBI). Differential expression of distinct clones was confirmed by RT-PCR. *In situ* hybridization showed that specific genes, such as S100 calcium-binding protein A4, nuclear factor of activated T cells 5, upstream of N-ras and F-box only protein 3, were also expressed predominantly in synovial tissues from patients with RA (3/7, 6/7, 5/7, 5/7, respectively), but not from normal individuals (0/3, 0/3, 1/3, 1/3).
Conclusion: SSH was a useful approach to compare the expression profile of cells under different conditions, and we could elucidate that distinct genes, including several novel genes, were differentially expressed in RA-SF during proliferation. Moreover, the expression of these genes could be found in RA synovium, especially at sites of invasion, suggesting that these molecules are involved in synovial activation in RA. It needs to be stressed, that the expression of certain genes in RA-SF depends on the stage of proliferation, and as such this stage needs to be considered in all analysis of differential gene expression in SF.
Acknowledgement: KM is supported by Japan Rheumatism Foundation, RM by Uehara Memorial Foundation and all others by their institutions.

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An approach to the analysis of gene expression in chronically activated T Lymphocytes

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We have been studying the intracellular signaling pathways in chronically activated T cells involved in effector responses and promoting the inflammatory process, and have been struck by the extent to which T cells stimulated with TNF for prolonged periods *in vitro* resemble RA synovial T cells. For example, TNF upregulates expression of the activation antigen CD69, induces non-deletional and reversible hyporesponsiveness to TCR ligation by uncoupling proximal TCR signalling pathways, and represses CD28 gene expression. We have explored the possibility that systematic expression profiling of murine T cell hybridomas stimulated with pM concentrations of TNF under controlled conditions might provide further insight into the phenotype and function of cytokine activated T cells, as well as the mechanisms through which TNF uncouples TCR signal transduction pathways. Expression profiling has been performed using medium density spotted arrays based on the Compugen™ gene set. This comprises 9,215 oligonucleotide 50-mer elements including housekeeping genes and "landing lights", and covers 7,524 known mouse genes (representing 17K RNAs and 303K ESTs). Genes whose expression are altered by TNF treatment have been identified by measuring the fluorescence ratio of Cy5- and Cy3-labeled target cDNA bound to each probe following hybridization of differentially labeled cDNA pools, prepared from TNF stimulated (Cy5) or control (Cy3) T cells. Based on this simplistic analysis, clusters of genes that appear to be differentially regulated in chronic TNF treated T cells have been identified and were found to include genes whose products may function to potentiate the inflammatory response. We report that the expression signature for chronic TNF stimulation suggests a phenotype which promotes cell survival, while enhancing Th1 differentiation, recruitment to sites of inflammation and effector responses. We are now analysing data from experiments designed to explore the possibility that this particular gene expression signature is distinct from the programme of gene transcription arising from short term TNF stimulation. We anticipate that this approach may provide further insight into the molecular mechanisms which promote chronic, as opposed to acute inflammatory responses.

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Efficacy of retroviral gene transfer into synovial fibroblasts is reduced by co-transduction with adenoviral vectors

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Objective: Virus-based gene transfer is an elegant method to overexpress molecules of choice and to analyze their effects on cartilage destruction in arthritis models. As combinations of different vector systems for delivery of two cartilage-protective genes may result in higher transduction efficacy, we compared double gene transfer, using adenoviral or retroviral vectors alone, to the combination of these two vectors.

Methods: RA synovial fibroblasts were transduced using IL-10- or IL-1ra-encoding MFG retrovirus (multiplicity of infection (MOI) of 50–200) and/or Ad5 adenovirus (MOI of 10–50). Double gene transduction was performed *in vitro* in a co-culture approach with (a) retroviral IL-10 and IL-1ra, (b) adenoviral IL-10 and IL-1ra, (c) adenoviral IL-10 and retroviral IL-1ra, (d) retroviral IL-10 and adenoviral IL-1ra. Cytokine production was measured by ELISA. Expression of proto-oncogenes and cytokines before and after gene transfer was analyzed using a combination of RNA arbitrarily primed PCR (RAP-PCR) and cDNA expression array to determine virus-mediated molecular effects.
Results: IL-1ra and IL-10 overexpression performed either with retroviral or with adenoviral vectors resulted in an enhanced synthesis of these cytokines. Cytokine expression was substantially higher in adenovirally transduced than in retrovirally transduced fibroblasts (IL-1ra 317 vs. 39 pg/ml; IL-10 221 vs. 44 pg/ml). Double gene transfer of a combination of retrovirus- and adenovirus-encoded genes resulted in a predominant expression of the gene encoded by the adenovirus even when the retroviral transduction was performed first. Virus-related effects on gene expression using LacZ or EGFP were higher in adenovirus- (4% of the proto-oncogenes and cytokines) than in retrovirus transduced fibroblasts (2%).
Conclusions: The results of the study demonstrate that combination of retro- and adenovirus-based vector systems for double gene transfer into RA synovial fibroblasts does not result in enhanced synthesis of the respective gene products but in suppression of the retroviral gene transfer. In addition, the experiments reveal that for human gene therapy the higher efficacy of adenovirus-based vectors needs to be outweighed against the lower effects on general alteration of gene expression when retrovirus-based vector systems are used.

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Family collections for the analysis of common autoimmune disease genes in systemic rheumatic and inflammatory diseases

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At present, little is known about the influence of genetic factors on the appearance and development of human autoimmune diseases, including rheumatic and inflammatory diseases. Our aims are i) to provide the scientific community with the material required to study the genetics of these diseases, individually and in their relation to each other and ii) to contribute to these investigations. We therefore collected caucasian families with i) one index patient suffering from rheumatoid arthritis (RA), systemic scleroderma (SSc), relapsing polychondritis (rPC), Wegener's granulomatosis (WG) or Systemic Lupus Erythematosus (SLE), ii) at least one first degree relative suffering from the same or another rheumatic or autoimmune disease, iii) healthy first degree relatives. Blood samples from all family members were used to prepare and store plasma (or serum), DNA, and Epstein-Barr-Virus (EBV) transformed B cell lines. Clinical, immunological and genetic information of interest was documented in a database. Families of healthy people were collected for comparison. Up to now we have collected more than 100 families, mainly in Southwest Germany. According to the patient index there are now 59 with RA, 12 with SSc, 3 with rPC, 4 with WG and 24 with SLE. In addition we collected information and material from more than 200 patients with healthy relatives or unavailable families. In parallel we started to compare patients with "familiar" or "non-familiar" diseases and have already observed interesting differences. In patients with RA these differences concern sex distribution, the presence of rheumatoid factors and the age at disease onset. At present typing of HLA-DRB1 alleles is being performed.
Acknowledgement: Funded by the Federal Ministry of Education and Research (Kompetenznetz Rheuma) and the German Scleroderma Foundation.

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A polymorphism within the Transforming Growth Factor β1 gene is associated with ankylosing spondylitis (AS)

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Introduction: Genetic factors that predispose individuals to ankylosing spondylitis (AS) are not fully understood. Axial and sacroiliac joint fibrosis are characteristic of AS and the presence of TGFβ1 mRNA in AS sacroiliac joints raised the possibility that this cytokine might be implicated in this fibrosis. We have therefore examined a group of HLA B27 positive AS patients to investigate whether they could be prone to fibrosis based on overproduction of TGFβ1.
Methods: DNA from 132 AS patients, 113 healthy controls from the West of Scotland were compared. DNA covering the G/C polymorphic site at position +915 in the TGFβ1 gene was expanded by PCR and examined using sequence specific primers. Levels of mRNA from stimulated PBMC's from AS patients and controls were analysed using Taqman PCR. Serum TGFβ1 was measured by ELISA on acidified serum.
Results: Although no significant differences in allele frequency was seen between these two populations examination of genotype frequencies showed that the AS patients were more likely to have the GG genotype associated with high TGFβ1 production (78% versus 64%; $P < 0.01$, OR=2.0. 95% CI 1.2–3.5). In keeping with this predisposition, the median level of TGFβ1 in serum from AS patients was 6359 pg/ml (range 3266–9587 pg/ml) which was higher ($P < 0.02$) than the controls (median 4903 pg/ml: range 136–7488 pg/ml). The levels of mRNA from AS patients were higher than controls.
Conclusion: This is the first report of a link with a polymorphic site within the TGFβ1 gene in AS. An increased predisposition to high TGFβ1 production could provide insights into the aetiology to AS. Our data confirm that genes other than B27 may be involved in AS pathogenesis.

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The association of HLA-DR/DQ coding and QBP promoter allelic polymorphism with antiphospholipid antibody response in SLE

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To ascertain if the polymorphism of HLA-DR, DQA and DQB alleles and QAP and QBP promoters influences the production of aCL and anti-β2-GP1 in SLE. While the role of HLA-antigens in directing various autoantibody responses is relatively well known, the effect of promoters is less established. Sixty-five consecutive unrelated Slovenian SLE patients (all female, mean age±SD 36±8.3 years, mean follow-up 93 months) and 74 unrelated healthy adults were investigated. aCL and anti-β2-GP1 were determined by ELISA. The patients and controls were typed for DRB1, DQB1, QAP and QBP alleles by PCR-SSO, using the 12th IHW primers, probes and protocols. The subtyping of DQB1 alleles as

well as DQA1 typing was carried out with selected Dynal SSP primers. Allelic and deduced haplotypic frequencies in patients and controls were compared using Fisher's exact test. 32 (49%) and 16 (25%) of 65 SLE patients were positive for IgG, IgM and/or IgA aCL and anti-β2-GP1, respectively. The frequency of the DQB1*0202 allele was significantly higher in the aCL ($P = 0.001$) and anti-β2-GP1 ($P = 0.001$) negative patients than in controls. Conversely, the DQB1*0301 allele and its promoter QBP3.1 were underestimated in the aCL ($P = 0.06$) and anti-β2-GP1 ($P = 0.001$) negative patients compared with controls. The DQB1*0202 allele may have a preventive role in provoking autoimmune response against both tested aPL. While the DQB1*0301 allele and its promoter QBP3.1 were underestimated in anti-β2-GP1 negative patients, they did not seem to protect from β2-GP1 specific autoimmune response in SLE patients. In contrast, we have already observed positive correlation of anti-Ro antibody response with the DQB1*0202 allele and the significantly underestimated DQB1*0301 allele and its promoter QBP3.1 in the same group of anti-Ro positive SLE patients.

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Expression of PAD enzymes and occurrence of citrulline-containing proteins in human blood and synovial fluid cells

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Antibodies directed against citrulline-containing antigens are extremely specific for RA. The amino acid citrulline is not incorporated into proteins during protein synthesis. It is generated by post-translational modification of arginine residues by PAD (peptidylarginine deiminase) enzymes. We investigated the expression of PAD enzymes and the occurrence of citrullinated proteins in peripheral blood (PB) and synovial fluid (SF) cells. PAD types 1 and 3 were absent from the investigated cells, while PAD types 2 and 4 (also known as type 5) were present. In monocyte-derived macrophages PAD type 2 mRNA expression was at a similiar level as in monocytes, while PAD type 2 protein was increased. PAD type 4 mRNA expression was significant in monocytes and almost absent in monocyte-derived macrophages, while PAD type 4 protein levels were similar. In monocytes no citrullinated protein could be detected, while in monocyte-derived macrophages citrullinated vimentin, which is (part of) the Sa-antigen, was present. A similar pattern of mRNA and protein expression was observed in mononuclear cells in paired PB and SF samples of RA patients. These results suggest that PAD type 2 is involved in the citrullination of SF proteins during inflammation.

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Two promoters for the CD5 gene: one operating in T cells and activated B cells and another restricted to resting B cells

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The CD5 T cell marker is present on a minute fraction of B cells. These B lymphocytes produce multispecific autoantibodies and generate most of the B chronic lymphocytic leukemias (CLL). However, very little is known regarding the regulation of the gene activity in B cells, compared with T cells.

Material and methods: B cells were isolated from tonsils, CLL blood and the control Daudi cell line cells, while T cells were obtained from normal peripheral blood (PB) and the control Jurkat cell line. Conventional and quantitative RT-PCR, 5' rapid amplification of cDNA ends (RACE), sequencing, Southern blot and *in situ* hybridization were required in this study.

Results: CD5 transcripts were identified in tonsil and CLL B cells, as well as PB and Jurkat T cells. Using the RACE technique, the 5' region of CD5 cDNA was amplified through an adaptor-ligated 5' primer coupled with a 3' end-specific primer. In these conditions, the conventional exon 1 was not identified in the mRNA from resting B cells. An alternative exon 1 was identified and its transcription confirmed using RT-PCR with appropriate primers and Southern blot. Importantly, the CD5 5'-flanking region contains TATA and CAAT boxes, and recognition sites for Ikaros in the B cells, but not in T cells. Furthermore, after a 48-hour stimulation with PMA, the conventional exon 1 was used in activated B- as in the T-cells.

Conclusions: Alternative exon 1 structures may initiate transcription of CD5 using two different promoters, one being operative in the resting B cells and the other in any T cells and B cells only when activated. Such a finding substantiates our hypothesis of innate (resting ?) and acquired (activated ?) $CD5^+$ B cells, which might be relevant to the pathogenesis of nonorgan-specific autoimmune disorders.

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Reshaping the shared epitope hypothesis: HLA-associated risk for rheumatoid arthritis is encoded by amino acid substitutions at position 67 to 74 of the HLA-DRB1 molecule

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Objective and methods: To further analyze the association of HLA-DRB1 alleles with disease susceptibility in recent onset rheumatoid arthritis (RA), 167 caucasian RA patients and 166 healthy controls were typed for HLA-DRB1.

Results: The association of susceptibility to RA with the group of alleles encoding the shared epitope susceptibility sequences (SESS) was confirmed in recent onset RA. Among non-SESS alleles DRB1*07, *1201, *1301 and *1501 showed significant protective effects. Even after correction for the influence of SESS alleles, significant independent protective effects of DRB1 alleles were observed. Protective alleles share a third hypervariable region motif. Independent homozygosity effects were observed both for susceptibility and protective alleles.

Conclusion: Non-susceptibility alleles differ significantly regarding RA risk. Protective alleles show clear homology at positions 67–74, often encoding Isoleucine at position 67 or Aspartic acid at position 70. Both susceptibility and protective alleles show homozygosity effects. Based on these results and literature data, in order to incorporate differential risks among non-susceptibility alleles, we propose to reshape the shared epitope hypothesis to, "HLA-associated risk for rheumatoid arthritis is encoded by amino acid substitutions at position 67 to 74 of the HLA-DRB1 molecule".

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Genome-wide gene expression in experimental arthritis: defining new targets of chronic/destructive rheumatoid arthritis (RA)

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Genome-wide expression analysis using microarrays enables us to visualize activation of complex signaling pathways in the total genome of an organism upon biological, pharmacological and toxicological stimulus or during pathological conditions. Rheumatoid Arthritis (RA) is a complex multigenic disease with yet unknown ethiology, and consequently, suitable target for genomics approach. We have used a high density DNA filter array, containing 25,142 DNA sequences, that represents a condensed mouse genome to analyze gene expression in animal models of RA. Well-defined animal models were chosen in order to investigate clear relationships between disease activity and gene expression. We have identified a number of genes whose targeted deletion or insertion results in modification of disease progression. Gene deletion of, e.g. IL-6 prevents development of sub-chronic inflammation without modifying the acute inflammation in zymosan-induced-arthritis (ZIA). Similarly, gene deletion of FcγR prevents some aspects of chronic inflammation in antigen-induced arthritis (AIA). On the other hand, adenoviral mediated gene transfer of IL-4 completely inhibits progression into the destructive phase in collagen-induced arthritis (CIA). Therefore we have analyzed gene-expression profiles in the following conditions: A) IL-6−/− vs. WT/ZIA, B) FcγR −/− vs. WT/AIA, C) AdIL-4 vs. AdC/CIA. Possible candidate genes for (sub)chronic inflammation or destructive arthritis were defined by two-parameter and cluster analysis of the expression profile. Seventy-seven common candidates, possibly involved in sub-chronic or destructive arthritis, were defined. Many of these are genes not yet inferred to be involved in inflammation. Selected ESTs were further analyzed and one candidate was cloned as a full length gene. Investigation into gene function is in progress. This approach, combining DNA array technology with cloning and functional characterization of candidate genes, proves highly effective in defining novel targets in inflammatory/autoimmune diseases such as RA.

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Discovery of distinctive gene expression profiles in human arthritides by cDNA micro-array analysis

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A potentially powerful way to gain insight in the complex pathogenesis of rheumatoid arthritis (RA) and to classify arthritides has arisen from cDNA microarray technology, which provides the opportunity to determine differences in gene expression of a large portion of the genome in search of genes that are differently expressed between clinically diagnosed arthritides. Therefore, we studied the gene expression profile of synovial tissues from affected joints of patients with diagnosed RA (n=21) in comparison to those of patients with osteoarthritis (OA) (n=9), a degenerative joint disease. Cy-5 labeled mRNAs from these samples were hybridized together with a Cy-3 labeled common reference mRNA preparation to arrays containing 18,000 genes of importance in immunology. The results revealed 1066 genes with a twofold difference in expression in at least 4 samples, relative to the median Cy-5 to Cy-3 ratio. Hierarchical cluster analysis revealed a remarkably ordered variation in gene expression profiles in the affected joint tissues of patients with RA and OA. These data revealed biological pathways and novel genes involved in disease. Based on the molecular signatures at least two distinct subsets of RA tissues could be identified. One class revealed abundant expression of gene clusters indicative of the presence and activation of the adaptive immune response, and the other group resembled the expression pattern of the OA tissues, which is characterized by a low inflammatory gene expression signature and increased tissue remodeling. The differences in the gene expression profiles reflect important aspects of biological variation within the clinically diagnosed arthritides that may help to understand the molecular pathology of and (sub-)classify rheumatic diseases.

Inflammation

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TNF-α blockade in early rheumatoid significantly reduces serum VEGF and ultrasonographic measures of synovitis and joint vascularity by 18 weeks

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This study compared the ability of ultrasonographic methods and serological measurement of vascular endothelial growth factor (VEGF) to discriminate between early rheumatoid arthritis (RA) patients receiving infliximab or placebo infusions added to pre-existing methotrexate (MTX) treatment over the first 18 weeks of therapy. Twenty-four patients with early RA (<3 years duration) on stable doses of methotrexate were randomised in a double-blinded study to receive infusions of infliximab (5 mg/kg) or placebo at entry then weeks 2, 6, and 14. At baseline and 18 weeks blood was taken and serum stored. At the same time points, metacarpophalangeal joints were imaged over the dorsal surface in longitudinal and transverse planes by high resolution ultrasound (HRUS) and power Doppler to assess synovial thickness, the presence of synovial vascularity and the number of vascularised erosions. At 18 weeks, there was a median reduction in DAS28 of 1.21 from baseline in the infliximab group and 0.39 in the placebo group ($P=0.157$). In the infliximab group 54% achieved ACR20 responses versus 18% in the placebo group ($P=0.08$). In contrast, median reduction in synovial thickness as assessed by HRUS was 50% in the infliximab group as compared with an increase of 1.2% in the placebo group ($P=0.014$). Median colour Doppler area diminished by 98.4% in the infliximab group as compared with a reduction of only 30.7% in the placebo group ($P=0.017$). The total number of vascularised erosions decreased by a median of

1.0 in the infliximab group with no change from baseline in the placebo group ($P = 0.001$). Median serum VEGF was reduced by 31.5% in the infliximab group and 3.1% in the placebo group ($P = 0.007$). In this cohort, changes in serum VEGF and sonographic measures of synovial thickening and joint vascularity showed a marked reduction in the infliximab treated group compared with the placebo and methotrexate treated group. These findings indicate that reversal of inflammatory and joint destructive mechanisms are already apparent at an early stage of treatment with infliximab.

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Expression of galectin-3 in rheumatoid arthritis synovium

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Background: Galectins are involved in cell-cell interactions, cell adhesion to extracellular matrix, tissue remodelling, cell growth and regulation of apoptosis. Particularly, galectin-3 has antiapoptotic properties, proinflammatory and chemotactic activities. An altered expression has been associated with tumor progression. The aim of this study was to investigate the expression pattern of galectin-3 in rheumatoid arthritis (RA) synovial tissues.
Material and methods: Synovial tissues were obtained after joint replacement from patients with RA (n=7) or osteoarthritis (OA, n=3). Specific sequences of galectin-3 cDNA were amplified by RT-PCR and used for the generation of digoxigenin-labeled riboprobes. *In situ* hybridisation was performed on paraffin sections. Immunohistochemistry was applied on paraffin and snap frozen sections using mouse monoclonal anti-galectin-3 antibodies. For comparison, the macrophage marker CD68 was used.
Results: In RA, galectin-3 was found at sites of joint destruction, as well as in the lining and sublining layers. The percentage of positive cells, however, was lower in the lining than in the sublining layer. A predominant expression of galectin-3 was found in cells with follicle-like structures and in perivascular infiltrates, whereas vessels remained negative. Synovial fibroblasts also stained positive and, at least in the sublining, the expressions of galectin-3 and CD68 appeared mutually exclusive. In contrast, in OA synovial tissues, only a few cells in the lining layer were positive for galectin-3. Similar results were obtained by *in situ* hybridisation and immunohistochemistry.
Conclusion: Taken together, theses observations suggest that galectin-3 is involved in cell-cell and cell-matrix interactions in the RA synovium and therefore may contribute to both the inflammatory and the destructive processes.

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Bacterial peptidoglycan stimulates integrin expression and MMP production of synovial fibroblasts

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Bacterial products such as peptidoglycan (PGN) have been found in joints of patients with rheumatoid arthritis. Recently, it has been shown that intra-articular injection of bacterial PGN can induce a transient arthritis in mice, indicating a possible role of bacterial products in the pathogenesis of arthritis. Whereas the activation of macrophages by PGN is established, it is not known whether synovial fibroblasts are also able to respond. We studied the activation marker expression of human synovial fibroblasts in culture after incubation with or without PGN *in vitro*. Cultured human synovial fibroblasts derived from RA patients were incubated in presence or absence of PGN. After culture periods of 24 to 48 hours the surface expression of integrins was measured by FACS using directly labeled antibodies. Expression of various matrix metalloproteinases (MMP) was determined by real time PCR (TaqMan).
PGN resulted in an upregulation of the surface expression of CD54 (ICAM-1) as compared to untreated cultures. In the tested cultured RA synovial fibroblasts the upregulation in responders ranged between 20% and 100%. PGN also upregulated the expression of MMP-3 and MMP-1 mRNA. These results suggest that the presence of bacterial PGN can activate synovial fibroblasts, to express ICAM-1 and MMPs. This activation might represent an important early step in the development of inflammatory arthritis.

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Stromal cell derived factor-1 (CXCL12) induces cell migration into lymph nodes transplanted into SCID MICE. An investigation of lymphocyte migration to secondary lymphoid organs

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SDF-1 (CXCL12), a CXC chemokine, has a primary role in signalling the recruitment of haematopoietic stem-cell precursors to the bone marrow during embryonic development. In post-natal life, SDF-1 is widely expressed and is induced in chronically inflamed tissues such as psoriatic skin and the rheumatoid synovium, but has also been implicated in the migration of lymphocytes to lymphoid organs. To investigate the role of SDF-1 in recirculation and homing *in vivo* we have developed a model in which human peripheral lymph nodes (huPLN) are transplanted into SCID mice. We have shown that huPLN transplants are viable and are vascularised by the murine circulation, forming functional anastomoses with transplant vessels. In addition grafts retain some of the histological features of the pretransplantation tissue, such as follicular dendritic cell-associated B-cell aggregates, lymphatic and HEV markers. We also show that SDF-1 is capable of inducing the migration of an SDF-1 responsive cell-line (U-937) and human PBL's from the murine circulation into the grafts in a dose dependant manner which is inhibitable by CXCR4 blockade. The mechanism of action of SDF-1 in this model is independent from that of TNF-α and does not rely on the upregulation of adhesion molecules (such as ICAM-1) on the graft vascular endothelium. This is the first description of huPLN transplantation into SCID mice, and of the functional effects of SDF-1 regarding the migration of human cells into huPLN *in vivo*. This model provides a powerful tool to investigate the pathways involved in cell-migration into lymphoid organs and potentially to target them for therapeutic purposes.

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Identification of homing peptides specific for synovial microvascular endothelium using *in vivo* phage display selection

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The microvascular endothelium (MVE) plays a major role in inflammation as well as tumour growth. Thus, the MVE represents an important therapeutic target. Peptide phage technology has been used *in vivo* to discover peptide sequences with binding capacity to organ specific MVE determinants in animals. The application of such powerful technology to humans has been limited by the obvious difficulties of performing phage-screening studies *in vivo*. By grafting human tissues into severe combined immunodeficient (SCID) mice, it is possible to target specifically human MVE determinants. Here we report for the first time the identification of synovial specific homing peptides by *in vivo* phage display selection in SCID mice transplanted with human synovium. Selected synovial homing peptide-phages were found to bind to human synovial graft MVE and retain their tissue homing specificity *in vivo* independently from phage component, disease origin of transplants and degree of human/murine graft vascularisation. In addition, the selected phages demonstrate tissue and species specificity in comparison to cotransplanted human skin grafts or mouse vasculature. Sequence analysis of the peptide inserts from synovial homing phages identified recurrent consensus motifs. One such motif maintains synovial MVE specificity both when expressed by a single phage-clone and as a free biotinylated synthetic peptide. Furthermore, the free peptide competes and inhibits, *in vivo*, the binding of the original peptide-phage to the cognate synovial MVE ligand. The identification of synovial homing peptides, with tissue and species specificity, may allow the construction of targeting devices capable of concentrating therapeutic/diagnostic materials to human joints.

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Inmunohistologic analysis of synovial tissue from early and late osteoarthritic patients. A potential role for COX-2 and NF-κB1 (p50) regulation in early disease

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Osteoarthritis (OA) is an erosive inflammatory disease originated by a biomechanical alteration that, in some patients, shows a strong component of inflammatory infiltrates in the synovial membrane resembling rheumatoid arthritis. T cells and macrophages initiate, amplify and perpetuate the inflammatory response. In stimulated cells, NF-κB, commonly formed by homodimers of NF-κB1 (p50), or heterodimers with RelA (p65) or c-Rel, bind to promoters and enhance, or occasionally inhibit, gene transcription through direct interaction with DNA. The activation of NF-κB may be a key step in the pathogenesis of OA. Inducible cyclooxygenase (COX-2) may also play a role in the inflamed profile, since it has a κB motive in its promoter. The aim of this study is to evaluate the differences in OA tissue, focusing on whether the pattern of NF-κB activation is quantitatively different in early and late stages, and its effect in COX-2 expression. Additional *in vitro* experiments, using OA cultured cells, were performed to evaluate the role of IL-6 and PGE2 in NF-κB activation and COX-2 induction. A significant increase in inflammatory cell infiltrate and hyperplasia of synovium was a feature found in early OA tissue. NF-κB1 and RelA were detected in all the OA samples studied, with significant increases observed in early OA tissue ($P = 0.006$ and $P = 0.012$). In concordance with NF-κB1/RelA, COX-2 expression was increased in early OA. Activation of p50 and p65 subunits of NF-κB showed a positive correlation with COX-2 (r=0.6169 and r=0.6620, respectively) and inverse correlation with COX-1 (r=−0.627 and r=−0.858) in early OA tissue, while only a positive correlation was observed between p50 and COX-2 in late OA (r=0.4129). *In vitro* synoviocytes cell cultures the activation of NF-κB in cells was observed together with an increase in COX-2 production. This activation was inhibited by parthenolide, an inhibitor of IκB degradation, and a concomitant decrease in COX-2 protein was observed as a result of the NF-κB inhibition. These findings support the conclusion that NF-κB and COX-2 play an important role in the early stages of OA, and specific inhibition could be a strategic approach in early OA.

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Comparison of knee joints with small joints: implications for pathogenesis and evaluation of treatment in rheumatoid arthritis

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Objective: Serial synovial biopsy samples are increasingly used for the evaluation of novel therapies for rheumatoid arthritis (RA). Most studies have used knee biopsies, but technical improvements have made serial small joint arthroscopy feasible as well. Theoretically, there could be differences in the features of synovial inflammation between various joints as a result of mechanical factors, differences in innervation, and other factors. Therefore, we compared the cell infiltrate in paired synovial biopsies from inflamed knee joints with inflamed small joints obtained simultaneously in RA patients.
Materials and methods: Nine RA patients with both an inflamed knee joint and an inflamed small joint (wrist or metacarpophalangeal) were subjected to an arthroscopic synovial biopsy of both joints on the same day. Multiple biopsy specimens were collected and stained for macrophages, T cells, plasma cells, fibroblast-like synoviocytes, and interleukin(IL)-6 by immunohistochemistry. Sections were analyzed by digital image analysis.
Results: The mean cell numbers for all investigated markers were equivalent in the samples from knee joints compared with the paired small joint samples. Statistical analysis by nonparametric tests identified no significant differences. Using Spearman Rank tests, we found significant correlations for the number of sublining macrophages (rho 0.817, $P < 0.01$), the number of T cells (rho 0.683, $P < 0.05$), and the number of plasma cells (rho 0.766, $P < 0.02$) when knee joints were compared with small joints. There was, however, no significant correlation for lining macrophages and fibroblast-like synoviocyte hyperplasia when large and small joints were compared.
Conclusion: The results presented in this study show that inflammation in one inflamed joint is generally representative for the process in other joints. Therefore, it is possible to use serial samples from the same joint selecting either large or small joints for evaluation of antirheumatic therapies. Hyperplasia of the intimal lining layer due to accumulation of intimal macrophages and fibroblast-like synoviocytes appears to depend in part on local factors.

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Expression of the EGF-TM7 family members EMR-2 and CD97 in rheumatoid synovial tissue

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Background: Fibroblast-like synoviocytes (FLS) express decay-accelerating factor (CD55) at high levels. One of its ligands, CD97, is a member of the EGF-TM7 family, a group of class B seven-span transmembrane receptors, which are prominently expressed by activated immune cells. Previous work has suggested a close association between $CD55^+$ FLS and $CD97^+$ intimal macrophages in rheumatoid arthritis (RA) synovium. These previous studies were performed with an antibody that binds both CD97 and EMR2, which is another member of the EGF-TM7 family. Recently, monospecific antibodies against EMR2 and CD97 were developed.
Objective: To determine the expression of CD97 and EMR2 using novel, monospecific antibodies to provide more insight into the factors that might be involved in leukocyte activation in rheumatoid synovial tissue.
Methods: Synovial tissue samples were obtained by arthroscopy from 19 RA patients, 17 inflammatory osteoarthritis (OA) patients, and 11 reactive arthritis (ReA) patients. Immunohistologic analysis was performed using the following antibodies: CLB-CD97/1 (which recognizes both CD97 and EMR2), CLB-CD97/3 (specific for CD97), and 2A1 (specific for EMR2). Bound antibody was detected according to a 3-step immunoperoxidase method. In addition, double immunofluorescence was performed. Stained sections were analyzed by digital image analysis using a standardized program and compared by nonparametric statistical analysis.
Results: CD97 was shown to be expressed on activated leukocytes in the intimal lining layer and in the synovial sublining in all forms of arthritis. Of interest, we observed a specific increase in the expression of EMR2 positive cells of myeloid lineage in RA compared with ReA and OA synovium, even after correction for cell numbers. These differences were statistically significant (RA versus ReA and OA for both lining and sublining: all P values < 0.03). Double immunofluorescence revealed that 40–60% of the macrophages in RA synovium expressed EMR2.
Conclusion: The increased expression of various members of the EGF-TM7 family in inflamed synovial tissue suggests a role in the formation of the architecture of the intimal lining layer as well as in the maintenance and amplification of synovial inflammation. EMR-2 might be involved in the specific activation of macrophages in RA.

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IgG-mediated activation of leukocytes is independent of Fc-γ receptor polymorphism

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Introduction: Ligation of Fc-γ receptors for IgG (FcγR) can trigger potent effector cell responses. Genetic polymorphisms of these receptors modify IgG binding, and influence internalization of immune complexes. In patients with infectious or autoimmune diseases, skewing towards low-binding FcγR alleles has been demonstrated. The objective of this study was to investigate the influence of FcγR polymorphism on leukocyte activation.
Methods: We analyzed activation of neutrophils and monocytes stimulated by aggregated or solid phase-coated IgG1, IgG2, and total IgG. Neutrophil donors were selected based on their FcγR genotype and homozygous for either FcγRIIa-H131/FcγRIIIb-NA1/1(HH-NA1/1) or FcγRIIa-R131/FcγRIIIb-NA2 (RR-NA2/2). Monocyte donors were homozygous for either FcγRIIa-H131/FcγRIIa-V158 (HH-VV) or FcγRIIa-R131/FcγRIIIa-F158 (RR-FF). Binding of immunoglobulins to lymphocytes was determined by flow cytometry. Activation of neutrophils was measured as the production of reactive oxygen intermediates (ferricytochrome c reduction), degranulation (lactoferrin release), and cytokine production (IL-8). TNF-α secretion was used as a measure of monocyte activation.
Results: IgG1 aggregates firmly bound to neutrophils of both types of donors, albeit more avidly to donors expressing HH-NA1/1 alleles. In contrast, IgG2 aggregates firmly bound to HH-NA1/1 FcγR neutrophils only. This binding could be blocked by preincubation of neutrophils with FcγRIIa and FcγRIIIb blocking antibodies. Despite the differences in binding of IgG subclasses to HH-NA1/1 and RR-NA2/2 neutrophils, we observed no differences in their activation. Activation of both types of neutrophils with IgG1 or IgG2 aggregates could be at least partially blocked by the addition of FcγR blocking antibodies. Similar to neutrophils, HH-VV and RR-FF monocytes were not distinguishable in their response to IgG, IgG1, and IgG2 as measured by TNF-α release, although RR-FF monocytes did not bind IgG2 complexes.
Conclusion: Although IgG-mediated activation of leukocytes is dependent on FcγR, it does not appear to be influenced by FcγR polymorphisms. These results are in favour of a new mechanism for IgG-mediated leukocyte activation, in which a short interaction between IgG and FcγR is sufficient to generate an appropriate inflammatory response. This may have important implications for inflammatory responses in infectious and autoimmune diseases.

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Overexpression of the autoantigen hnRNP-A2 (RA33), the tumour suppressor p53 and activated MAP-Kinase p38 in inflamed synovial tissue

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Objective: Overexpression of the nuclear autoantigen hnRNP-A2 (RA33) has recently been observed in synovial tissue of RA patients and TNF transgenic mice. To further investigate this issue expression of hnRNP-A2 was compared with that of two other hnRNP proteins, the closely related hnRNP-A1 (a rare autoantigen in RA) and the structurally different hnRNP-C (which is not an autoantigen). In addition, expression of the tumour suppressor p53 and the MAP-kinase p38 was studied.
Methods: Synovial tissue of patiens with RA or osteoarthitis and specimen from patients with early arthritis of <1 year duration were analyzed by immunohistochemistry
Results: HnRNP-A2 was highly overexpressed in RA synovial tissue as compared to tissue of osteoarthritis patients, and most abundantly found in CD68-positive cells of the lining layer. The antigen was not only localized in the nucleus but also in the cytoplasm confirming previous observations. Nuclear overexpression was also observed for hnRNP-C, whereas expression of hnRNP-A1 appeared normal. Remarkably, in the majority of hnRNP-A2 expressing cells also the p53 tumor suppressor was overexpressed and aberrantly localized in the cytoplasm. Furthermore, the MAP-kinase p38 was acitvated in these cells as revealed by a monoclonal antibody specifically recognizing the phosphorylated (activated) form of this kinase. This indicated that cells overexpressing hnRNP-A2 and p53 had been activated by

proinflammatory cytokines such as TNF or IL-1. A comparable result was obtained with tissue from patients with early arthritis, irrespectively of their diagnosis (RA or reactive arthritis). So far, the conditions that lead to aberrant expression of these proteins have not been clearly defined since even prolonged exposure of macrophages or synovial fibroblasts to TNF or IL-1 did not cause any changes in hnRNP-A2 expression or induce its cytoplasmic accumulation. This was only achieved by treatment with the RNA polymerase II inhibitor actinomycin D which subsequently led to apoptosis and accumulation of hnRNP-A2 in apoptotic bodies.
Conclusion: The state of chronic inflammation in the rheumatoid synovium seems to cause aberrant expression and/or modification of (some) proteins which may lead to loss of tolerance and induction of patholocigal autoimmune reactions in genetically susceptible individuals.

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HMGB1 is a potent proinflammatory mediator expressed abundantly in chronic synovitis

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Aim: To dissect the role of the endogenous, cytokine-like protein high mobility group 1 protein (HMGB1) in arthritis, we set out to investigate the presence of HMGB1 in synovial biopsies from rats with adjuvant arthritis and in synovial biopsies and synovial fluid samples from patients with active RA.
Background: HMGB1 is a DNA-binding, non-histone, nuclear protein present in all nucleated cells. Previous results have demonstrated that HMGB1 is released from the cytoplasm of activated monocytes and macrophages and that extracellular HMGB1 is a potent inducer of production of proinflammatory cytokines in monocytes and macrophages. Furthermore, anti-HMGB1 treatment inhibits LPS-induced lethality in sepsis in mice.
Methods: Presence of released HMGB1 in synovial membranes from arthritic rats and synovial membrane biopsies from RA patients, were detected by immunohistochemistry. Analysis of HMGB1 in synovial fluid was performed by western blotting.
Results: In rat ankle joint specimens obtained at the onset of arthritis, as well as in the chronic stage of the disease, we detected cytoplasmic HMGB1 in macrophage/monocyte-like cells in the synovial membrane as well as in synovial fluid. The levels of cytoplasmic expression was higher at later stages of disease. A similar picture was obtained when immunohistochemical stainings were performed on synovial biopsies from RA patients. Analysis of synovial fluid samples from RA patients revealed high levels of released HMGB1. Intra-articular rHMGB1 injections in rats caused erosive synovitis.
Conclusion: HMGB1 is a newly identified proinflammatory molecule, now shown to be present in arthritic joints of both RA patients and rats with adjuvant arthritis. With reference to the previously demonstrated capacity of HMGB1 to stimulate the production of TNF and IL-1β, we speculate that HMGB1 could be of importance in the pathogenesis of arthritis.

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Expression of protease-activated receptors in arthritic synovial tissues

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Clinical and experimental evidence suggests that synovial thrombin formation in arthritic joints is prominent and deleterious, leading to exacerbation of rheumatoid arthritis (RA). In this context, cellular effects of thrombin mediated by the protease-activated receptors (PARs) in arthritic joints may be of paramount significance. Four PARs have now been identified. PAR1, PAR3, and PAR4 can all be activated by thrombin whereas PAR2 is activated by trypsin and few other proteases.
We first explored PARs expression in RA synovial tissues. Synovial membranes from 11 RA patients were analyzed for PARs expression by RT-PCR and by immunohistology. PAR4 was found in all the biopsies, whereas the expression of PAR1, PAR 2 and PAR3 was more restricted (8/11, 5/11 and 3/11 respectively). In the arthritic synovial membrane of murine antigen-induced arthritis (AIA) we found coexpression of the four different PARs. Next, we explored the functional importance of PAR1 during AIA *in vivo* using PAR-1 deficient mice. The phenotype of PAR1-deficient mice (n=22), based on the analysis of arthritis severity (as measured by 99m tecnetium uptake, histological scoring and intra-articular fibrin measurements) was similar to that of wild-type mice (n=24). In addition, the *in vivo* production of antibodies against mBSA was also similar. By contrast, the mBSA-induced *in vitro* lymph node cell proliferation was significantly decreased in PAR1-deficient mice as compared with controls. Accordingly, mBSA-induced production of interferon-γ by lymph node cells in culture was significantly decreased in PAR1-deficient mice as compared with controls, whereas opposite results were observed for production of IL-10.

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IFN-β-induced IL-1Ra synthesis in human monocytes involves PI 3-kinase-STAT1 signaling pathway

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IFN-β displays an anti-inflammatory property by inducing IL-1Ra without triggering synthesis of IL-1β in human monocytes (Mo). IFN-β initiates JAK-STAT pathway that may cross-talk with components of MAP- and PI 3-kinase pathways. Since maximal activation of transcription by several STATs requires both Tyr and Ser phosphorylation, we investigated the role of MAP- (ERK1/2) and PI 3-kinases in IFN-β-induced IL-1Ra production in Mo. The PI 3-kinase inhibitor Ly294002 but not the MAP kinase inhibitor PD98052 suppresses, in a dose-dependent way, IL-1Ra production in Mo at a protein level correlating with the reduction of steady state levels of IL-1Ra mRNA. IFN-β treatment of Mo leads to rapid Ser-phosphorylation and nuclear translocation of STAT1 that is inhibited by Ly294002. Interestingly, suppression of PI 3-kinase activity in Mo stimulated by IFN-β and anti-CD11b mAb results in inhibition of IL-1Ra and upregulation of IL-1β production, suggesting that PI 3-kinase might be a check-point signaling molecule favoring IL-1Ra synthesis. Involvement of PI 3-kinase pathway in IL-1Ra synthesis seems to be independent of the differentiation state of Mo: M-CSF differentiated Mo requires activation of PI 3-kinase to synthesize IL-1Ra following IFN-β treatment. Thus, IFN-β induced IL-1Ra production in Mo by simultaneously activating components of JAK-STAT and PI 3-kinase signaling pathways.

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Activating FcγRIII determines cartilage destruction during immune complex arthritis but not in the presence of T-cell immunity

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Introduction: We have recently shown that activating Fcγ receptors determine metalloproteinase (MMP)-induced cartilage

destruction, seen in various murine models of arthritis mediated by immune complexes (IC). In the mouse, two activating FcγR (FcγRI and FcγRIII) which bind IC have been described. In this study, we investigated the role of activating FcγRIII in MMP-mediated cartilage destruction in two different models of experimental arthritis, one induced only by ICs and the second by ICs and T cells.

Methods: Mice made deficient for FcγRIII and their wildtype controls(C57BL/6) were used. Immune complex arthritis (ICA) was induced by injecting lysozyme directly into the knee joint of mice, which had previously been given antilysozyme intravenously. T cell-mediated IC-mediated IC-arthritis (AIA) was induced by immunizing mice with mBSA in CFA and injection of the antigen directly into the knee joint, 3 weeks after immunization. Cartilage destruction was studied by immunolocalisation of MMP-mediated neoepitopes (VDIPEN) and matrix erosion in total knee joint sections. Adaptive cellular cells (T cells) and humoral immunity (anti-mBSA antibodies) were investigated using lymphocyte stimulation assay and ELISA.

Results: ICA induced in naive C57BL/6 knee joints showed florid inflammation at day 1 and 3. MMP-mediated cartilage destruction and matrix erosion was moderate at day 3. When ICA was induced in knee joints of FcγRIII deficient mice, joint inflammation, MMP-mediated cartilage destruction and erosion was significantly lower (respectively 95, 100 and 80%). In addition, AIA was induced in FcγRIII–/–. Immunisation of FcγRIII–/– did not alter adaptive cellular immunity or humoral immunity against mBSA if compared to wildtype controls. Induction of AIA showed similar swelling at day 1, 3 and 7. At day 7, histology showed severe chronic joint inflammation not different from controls. MMP-mediated cartilage destruction and erosion was also similar to wildtype controls. In contrast, AIA induction in knee joints of Fcg chain–/– (which lack both functional FcγRI and III), MMP-mediated cartilage destruction and erosion was completely prevented.

Conclusion: FcγRIII is the dominant activating receptor in MMP-mediated cartilage destruction and erosion during arthritis merely induced by immune complexes. In T cell driven immune complex arthritis, however, FcγRIII is not important or redundant.

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Sustained high expression of Fcγ receptor II (CD32) on dendritic cells (DCs) in patients with rheumatoid arthritis (RA)

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Introduction: DCs are proffesional antigen presenting cells. For antigen internalisation, DCs use the Fcγ receptor II (CD32) which recognises anti-IgG-complexes and therefore might be involved in regulation of autoimmunity. Normally, CD32 is abundantly expressed on immature DCs and down-regulated after full maturation.

Objective: Evaluation of CD32 expression on DCs from RA patients.

Methods: Peripheral blood mononuclear cells obtained from RA patients and healthy donors were cultured with GM-CSF and IL-4 for 6 days to obtain immature DCs. Stimulation with LPS from day 6 yielded mature DCs at day 8. The expression of surface markers involved in T cell-DC interaction (DC-SIGN, CD83) antigen uptake (FcγRI, II and III) and presentation (CD80, CD86, MHC-I and MHC-II) were studied using FACS analysis. In addition, RT-PCR and *in vivo* colocalisation studies using DC-specific markers in synovium were used to confirm these findings.

Results: In both RA patients and controls DCs expressed expected levels of CD80, CD86, CD83, MHC-I, MHC-II and DC-SIGN. Interestingly, immature DCs showed a threefold increase in expression of CD32 when compared with controls (mean fluorescence 185.8 vs. 63.0 $P < 0.01$). Moreover, this increased expression of CD32 is sustained in fully mature DCs from RA patients (mean fluorescence 452.3 vs. 187.8 $P = 0.005$), whereas CD32 expression is strongly down-regulated upon maturation of DCs from controls. The sustained high expression of CD32 was further confirmed by RT-PCR. Both FcγRIIa and FcγRIIb were increased in immature DCs as well as mature DC from RA patients but not in DCs from healthy controls. In RA both immature and mature DCs showed a balance towards FcγRIIb. Colocalisation between FcγRII and DC-LAMP and DC-CK1 was clearly observed in RA synovial tissue.

Conclusion: Both immature and mature DCs from RA patients showed a significant increased expression of the FcγRII suggestive for an aberrant maturation pathway. *In vivo*, mature DCs with a high FcγRII expression are clearly present in the synovium in RA. These findings are suggestive for a crucial role of high FcγRII expression on DCs in synovial tissue in promoting and sustaining the local immune response in RA.

Cell signalling

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Signaling pathways involved in IL-18 induced VCAM-1 expression in RA synovial fibroblasts

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In a recent report, we suggested the implication of a phosphoinositide-3 kinase (PI3 kinase) pathway in IL-18-induced VCAM-1 expression in rheumatoid arthritis (RA) synovial fibroblasts. Here, we demonstrated for the first time that IL-18 rapidly activates PI3 kinase and its downstream effector Akt. In our investigation to elucidate the signaling events downstream and upstream of PI3-kinase, we found that IL-18 induces a parallel pathway involving extracellular regulated kinases (ERK) 1/2. Using specific kinases inhibitors LY294002 and PP2, we showed that ERK1/2 activation was independent of PI3 kinase but dependent of Src kinase with an approximately 63% ($P < 0.05$) decrease of phosphorylated ERK. IL-18 also showed a time dependent activation of both c-Src, Ras and Raf-1suggesting the implication of this signaling cascade in ERK activation. Electrophorectic mobility shift assay demonstrate that activator protein-1 (AP-1) is activated by IL-18 through ERK and Src but not through PI3 kinase. Finally, the Src kinase, which is known to activate PI-3 kinase may represent the early event in IL-18-activated PI3 kinase because the specific inhibitors of these two kinases both affect IL-18 induced VCAM-1 expression in RA synovial fibroblasts by 50% ($P < 0.05$). In contrast, PD98059 and pertussis toxin had no impact on VCAM-1 expression excluding the participation of ERK and Go/i proteins in the IL-18-mediated VCAM-1 production. These data demonstrate the activation of two novel pathways in IL-18-stimulated RA synovial fibroblasts and they indicate that it is possible to selectively inhibit the expression of VCAM-1. Targeting PI3 kinase may represent an interesting approach to the development of selective drugs in inflammation and RA.

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Effect of P38 mapkinase inhibitor RWJ-67657 on proinflammatory mediators produced by IL-1β- and/or TNFα-stimulated rheumatoid synovial fibroblasts

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Introduction: The p38-mitogen activated protein kinase (p38 MAPK) inhibitor RWJ-67657 has been shown to effectively suppress clinical and cytokine response to endotoxin in healthy human volunteers (Fijen JW: *JCI* 2001, **124**:16). In patients with rheumatoid arthritis (RA) p38-MAPK activity is observed in the synovial lining layer (Schett G, *Arthritis Rheum* 2000, **43**:2501). To further characterise the role of p38-MAPK in RA the effect of RWJ-67657 on IL-1β and/or TNFα induced mRNA expression and production of cytokines (IL-6 and IL-8) and matrixmetalloproteinases (MMP-1, MMP-3 and TIMP-1) as well as on mRNA expression of ADAMTS-4 (aggrecanase) and COX-2 was studied in cultured synovial fibroblasts.

Methods: Rheumatoid synovial fibroblasts were isolated from patients who underwent a total joint replacement. Cells at passage 3–8 were stimulated with 1 ng/ml IL-1β and/or TNFα with or without preincubation with 0.001–30 μM RWJ-67657. RNA isolation and RT-PCR was performed after 6 hour stimulation and protein levels in supernatants were measured by ELISA after 48 hour stimulation.

Results: RWJ-67657 induced a dose-related decrease in IL-6, IL-8 and MMP-3 production, both after IL-1β and TNF-α stimulation. Inhibition of MMP-1 was seen only at high levels of RWJ-67657. TIMP-1 was produced constitutively and was not affected by stimulation or inhibition. These findings were all confirmed by mRNA expression studies. COX-2 mRNA expression was induced both by IL-1β and TNFα and could be inhibited by RWJ-67657. ADAMTS-4 mRNA expression was only seen after IL-1β stimulation, which could be inhibited by RWJ-67657.

Conclusion: RWJ-67657 is a potent inhibitor of cytokine and MMP production, as well as of their mRNA expression in stimulated RA synovial fibroblasts. Also COX-2 and aggrecanase mRNA expression was inhibited by RWJ-67657. Thus, inhibition of the p38 MAPK pathway by RWJ-67657 effectively leads to inhibition of different inflammatory mediators produced by rheumatoid synovial fibroblasts. Therefore, RWJ-67657 could be of therapeutic significance in RA.

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Fibroblast-like synoviocytes from rheumatoid arthritis patients express less flice-inhibitory protein than FLS from osteoarthritis, trauma and prosthesis loosening patie

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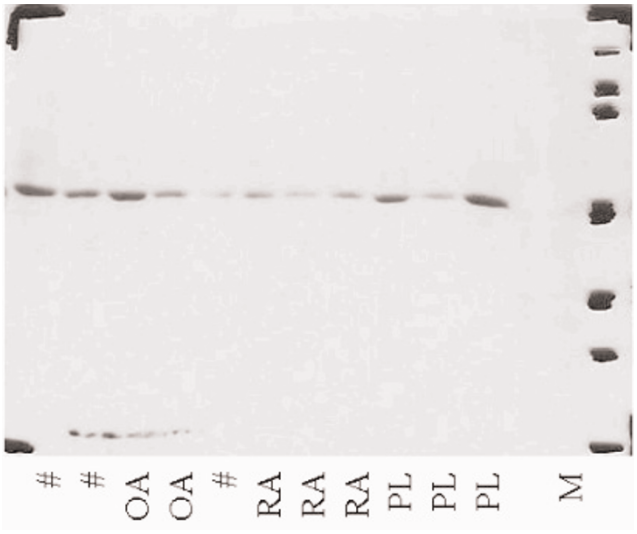
Rheumatoid arthritis (RA) is characterized by hyperplasia of the synovium and degradation of cartilage by fibroblast-like synoviocytes (FLS). The hyperplasia is thought to be a result of an imbalance between proliferation and apoptosis. Proliferation and apoptosis can be regulated by death receptors like TNF receptor and Fas. TNF is abundantly expressed in the rheumatoid synovium and could contribute to the hyperplasia by promoting proliferation and/or inhibition of apoptosis. In this study it was investigated whether overexpression of flice inhibitory protein (FLIP), a protein that inhibits death receptor mediated apoptosis, could potentially be involved in the hyperplasia of FLS. Epression of FLIP was investigated using a western blot and was seen in all samples tested, but most expression was seen in two trauma patients (n=3), one OA patient (n=2) and two patients with prosthesis loosening (n=3). RA patients (n=3) showed the least expression of FLIP (see figure 1). Thus, in RA FLS no overexpression of FLIP was observed, therefore it is unlikely that FLIP contributes to resistance to apoptosis of FLS and hyperplasia in the rheumatoid synovium.

Figure 1



Western blot showing expression of human FLIP in several patients. #, Trauma patients; RA, rheumatoid arthritis; PL, prosthesis lossening patients.

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Chronic exposure to TNF activates distinct TNF-R signaling pathways favouring cell survival in T lymphocytes

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Tumour necrosis factor α (TNF) is a multipotent cytokine. Its role in the pathogenesis of rheumatoid arthritis (RA) is now well established. However, the signaling pathways activated by TNF in the context of chronic inflammation are less well understood. We have previously reported that chronic as opposed to acute TNF exposure induces non-deletional and reversible T cell hyporesponsiveness to T cell receptor ligation. To explore this further, we set out to explore more precisely how TNF signals in T lymphocytes, and in particular to determine whether chronic exposure to TNF activates a cascade of signaling pathways which are qualitatively or quantitatively distinct from those activated following short term stimulation. Initial experiments revealed that the p55 TNF-R is necessary and sufficient to induce T cell hyporesponsiveness. Accordingly, we focussed subsequent studies on p55 TNF-R signaling in a murine T cell hybridoma model. Over 8 days, repeated stimulation of T cells with pM doses of TNF leads to chronic, stable cytokine exposure throughout the culture period. At this low dose NF-κB is activated, while activation of other p55 TNF-R signaling pathways is much less pronounced. Within minutes of TNF-R ligation, high dose (nM) TNF induces strong activation of NF-κB and JNK in

control T cells. In contrast, TNF-R ligation of T cells chronically exposed to low dose TNF leads to attenuation of JNK activation, while activation of NF-κB and ERK are spared. Preliminary data indicate that translocation of RelA to the nucleus is enhanced and sustained in TNF treated T cells. Together, the data indicate that the signaling pathways in T cells exposed to inflammatory cytokines for prolonged periods may be distinct from those activated by cytokines over much shorter periods. We speculate that in diseases such as RA, T cell effector responses may be promoted by selective activation of the ERK and NF-κB pathways, which promote T cell survival. The data also predict that the gene expression signature arising from chronic TNF stimulation should be distinct from the programme of gene transcription arising from short term TNF-R engagement.

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Prolonged stimulation by TNF uncouples T cell receptor (TCR) signalling pathways at many levels

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$CD4^+$ cells at sites of inflammation such as the rheumatoid joint exhibit profound proliferative hyporesponsiveness. We recently demonstrated that expression of the zeta chain of the TCR (TCR zeta) was down-regulated in TNF-treated T cells. Reduced expression of TCR zeta, together with impaired assembly and stability of the cell surface TCR/CD3 complex, may uncouple proximal signal transduction pathways from the TCR. We have proposed that signalling pathways downstream of the TCR may be attenuated as a result, and that this may contribute to the hyporesponsive phenotype. A T cell hybridoma cell line was generated which expressed a single-chain Fv/TCR zeta chimaeric receptor (C2-zeta) under control of retroviral LTR: TNF was not expected to affect expression of C2-zeta. We now report that the receptor-proximal tyrosine phosphorylation responses of these cells were reduced after prolonged TNF-treatment, even though C2-zeta expression was unchanged. Furthermore, while the amplitude and duration of calcium release from intracellular stores were slightly lower in TNF-treated T cells, there was a marked attenuation of capacitative calcium entry in these cells. In addition, PMA- and ionomycin-stimulated IL-2 production was also suppressed in cells that had undergone prolonged culture in TNF. These data indicate that chronic exposure to TNF attenuates a number of signal transduction events downstream of the TCR, independently of TNF-induced suppression of TCR zeta expression. Such changes may account, in part, for the profound hyporesponsiveness of T cells at sites of inflammation.

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Constitutively activated N-Ras stimulates LFA-1-dependent adhesion in rheumatoid arthritis synovial fluid T lymphocytes

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Synovial fluid (SF) T lymphocytes isolated from rheumatoid arthritis (RA) patients display constitutive activation of the integrin LFA-1. LFA-1-dependent T cell interactions with ICAM-bearing fibroblast-like synoviocytes and macrophages contribute to the inflammatory state of the joint via retention of T lymphocytes in the synovium and induction of inflammatory cytokines. SF components, notably TGF-β, are sufficient and required to sustain activation of LFA-1 in SF T lymphocytes, but little is known about the intracellular signalling pathways regulating LFA-1-dependent adhesion in SF T lymphocytes. We have recently found that deregulated signalling via the small GTPases Ras and Rap1 is responsible for chronic oxidative stress in SF T lymphocytes. Although activated Rap1 is a critical mediator of T cell receptor and CD31 -stimulated LFA-1 adhesion, SF T lymphocytes adhere via LFA-1 in the complete absence of Rap1 signalling. We find that both N-Ras and K-Ras isoforms of Ras are constitutively activated in SF T cells, and that transient expression of activated N-Ras, but not K-Ras or H-Ras, stimulates LFA-1-dependent adhesion in Jurkat T lymphocytes. Adhesion induced by N-Ras is insensitive to overexpression of the Rap1 negative regulatory protein RapGAP, consistent with the observation that SF T cells are highly adherant despite undetectable levels of activated Rap1. We provide pharmacological evidence indicating that N-Ras and Rap1 induce adhesion by distinct signalling mechanisms. Identification of N-Ras as a key intracellular signalling protein mediating lymphocyte retention in RA joints provides potential targets for new therapeutic strategies for RA.

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Potassium calcium-dependant BK channel: a potential membrane target of estrogens in human osteoblasts

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Estrogens have been recognized for many years for their positive effects on bone. However, their mechanisms of action are still only partially resolved. Although it is generally accepted that estrogens act at genomic level by binding to intracellular estrogen receptor, they trigger nongenomic effects mediated by plasma membrane receptor. One of these is to increase activity of the BK channel in many cells types (smooth muscle and vascular endothelium), but this has never been reported in bone. BK channels are composed of alpha (encoded by *Slo* gene) and beta subunits. The alpha subunit forms the K+/−selective pore, while beta subunits influence the pharmacology, kinetics, and voltage/calcium sensitivity of the BK channel. β1 and β4 subunits have been reported to mediate estrogen effects on the BK channel. In this study, we have shown that primary human osteoblasts, and 3 cell lines of human osteosarcoma (MG63, SaOs2, CAL72) express the Slo protein (western blot). Using RT-PCR, we observed in all these cells the expression of the 4 beta subunits (β1, β2, β3, β4). In electrophysiology analysis, we observed a calcium-induced potassium current of high conductance. As expected, β1 subunit expression induced Charybdotoxine and Iberiotoxine (classical inhibitors of the BK channel) resistance of the BK channel. We investigated whether 17β estradiol increased the probability of the BK channel opening (NP0). Our data indicate that 17β estradiol (5 nM) increase BK channel activity, shedding a new light on the nongenomic effects of estrogens and opening new ways for therapeutic development.

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Molecular mechanisms of resistance to disease modifying antirheumatic drugs (DMARDs)

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Background: Drug resistance is a common cause of treatment failure in infectious and neoplastic diseases. Relatively little is known about the molecular mechanisms of resistance to DMARDs.
Objective: To obtain insight into the onset and molecular mechanism(s) of resistance to 2 DMARDs: 1) the antimmalarial chloroquine (CHQ) and 2) sulfasalazine (SSZ), an inhibitor of the activation of NF-κB.
Methods: Human CEM (T) cells were used as an *in vitro* model system of a target cell in rheumatoid arthritis (RA). Resistance to CHQ and SSZ was provoked by growing CEM (T) cells in stepwise increasing concentrations of either of these DMARDs.
Results: Over a period of 5 months, CEM (T) cells developed a level of 4–5-fold resistance to CHQ and SSZ. The molecular basis of CHQ resistance appeared to be due to a 5-fold overexpression of one of the ATP-Binding Cassette (ABC) drug efflux proteins; the multidrug resistance-associated protein 1 (MRP1). Consistently, blockers of MRP1 (MK571 and probenecid) reversed CHQ resistance in CEM/CHQ cells. The molecular basis of SSZ resistance appeared to be due to a marked overexpression of another ABC protein; the breast cancer resistance protein (BCRP). A blocker of BCRP reversed resistance for SSZ in CEM/SSZ cells by more than 50%. Beyond this, CEM/SSZ revealed a diminished basal level of expression of cytoplasmic phosphorylated IκB-α and nuclear NF-κB(p65).
Conclusions: Members of the ABC family of drug efflux pumps (i.e. MRP1 and BCRP) can confer resistance to DMARDs (CHQ and SSZ, respectively). This result warrants further investigations into the contribution of drug efflux pumps in treatment failure of RA patients with DMARDs.

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Deregulated Ras and Rap1 signaling in rheumatoid arthritis T cells leads to persistent production of free radicals

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Background: We have previously found that key Ras family signaling molecules, H-Ras and Rap1, are deregulated in SF T lymphocytes: Ras is constitutively active, whereas Rap1 can not be activated. Here we report that similar signaling events in Jurkat cells lead to the persistent production of intracellular reactive oxygen species (ROS), mimicking the hyporesponsiveness of synovial T lymphocytes.
Methods: Jurkat cells were cotransfected with pCMV-CD20 expression plasmid to differentiate between transfected and untransfected cells and 20 μg expression plasmid encoding for RasV12, RasN17; RasV12/G37, RasV12/E38, RasV12/C40, RlfCAAX, RalV23, RalN; RapV12, RapGAP, RapN17 RasV12/G37, RasV12/E38, RasV12/C40. For ROS determination, cells were first stained with CyCr CD 20 for detection of positive transfected cells. CD20-pos cells were analysed on a FACScan for the mean fluorescence intensity of oxidated DCF (di-chlorofluorescin), as a measurement for the presence of intracellular ROS.
Results: 1) Treatment of either PB T lymphocytes or Jurkat cells with either anti-CD3 antibodies or PMA/I leads to a rapid and transient generation of intracellular ROS which was maximal 2–5 minutes poststimulation. 2) In Jurkat, expression of Ras is sufficient to induce the generation of ROS, in a Ral dependent manner and Ras is required for TCR and PMA/ionomycine induced ROS3. Consitutively active Rap1 suppressed TCR, TPA and Ras-induced ROS production in a PI3-kinase dependent manner, while inactivation of the Rap signalling pathway sensitized T cells to agonist-induced ROS generation.
Conclusions: 1) Rap1 GTPase plays a critical role in the termination of transient ROS production in T lymphocytes following agonist stimulation. This is unlikely due to competitive sequestration of Ras effectors by Rap1 as Rap1-dependent inhibition of ROS is PI 3-kinase-dependent, while Ras-induced ROS generation is Ral-dependent. 2) As the chronic oxidative stress observed in SF T cells can be mimicked by transient transfection of Jurkat cells with interfering Rap1 proteins, we hypothesize that deregulation of Rap and Ras are the critical events leading to the disturbed intracellular redox balance that underlies the hyporesponsiveness of Synovial T cells in rheumatoid arthritis.

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Induction of apoptosis by polyamine metabolites in immunocompetent cells and different tumor cell lines

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Polyamines (PAs) are involved in regulation of cell growth and cellular survival by interacting with processes like translation, transcription or ion transport. It is described that polyamines can induce apoptosis in mesenchymal cell lines. The aim of our study was to analyze whether the physiological PAs (putrescine, spermidine or spermine) or the PA-derivate deoxyspergualin (DSG), a novel immunosuppressant, induce apoptosis in immunocompetent cells. Furthermore, we wanted to investigate which molecular mechanisms are involved in the execution of the cell death program. By means of flow cytometric analysis we found an induction of apoptosis by spermine (Spm) and DSG in quiescent and activated PBMCs, PHA generated lymphoblasts, and various tumor cell lines (Jurkat, SKW-3, U937). Moreover, DSG and Spm triggered apoptosis in human Fas-deficient cells and in cell lines MV 4.11. and RS 4.11., which are described to be resistant to apoptosis induction by many conventional chemotherapeutic agents. Apoptosis induction after Spm or DSG treatment was dependent on caspase activity and associated with a decrease in mitochondrial membrane potential and Bcl-2 expression. In order to test whether PAs mediate their proapoptotic effects through metabolites resulting from PA catabolism, we tested different antagonists of PA degrading enzymes or PA metabolites. We show that aminoguanidine (inhibition of PA oxidase), aldehyd-dehydrogenase (degradation of PA-aldehydes) or N-acetyl-cystein (prevention of PA-induced glutathion depletion) prevented PA-mediated apoptosis. Acrolein, and PA-related aldehyde, could induce programmed cell death in our system. We conclude that PA aldehydes and PA-triggered glutathion depletion cause apoptosis in immunocompetent cells and apoptosis-resistant tumor cell lines.

Cytokines

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TRAIL-mediated enhancement of collagen production by human lung fibroblasts

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family that induces apoptosis in a variety of transformed cell lines and in normal human hepatocytes *in vitro*. 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 CD95 ligand, a receptor of which may not only mediate apoptosis of T cells, but also mediate the proliferation of normal human fibroblasts. Considering structural and functional similarities between TRAIL and CD95 ligand, we examined the effects of soluble TRAIL on normal human lung fibroblasts. Collagen α2(I) mRNA expression was assessed by real-time RT-PCR, with ribosomal protein S9 as an internal standard. Total soluble collagen was measured in culture supernatants using the Sircol Biocolor Assay. Both normalized α2(I) collagen mRNA expression and total soluble collagen secretion were increased upon TRAIL stimulation, with peak response (>5-fold increase) at 10 ng/ml TRAIL. DNA microarray hybridization revealed 78 genes involved in signal transduction, DNA transcription, and tissue remodeling, whose expression level increased, and 8 genes whose expression level decreased >2.5-fold in comparison with quiescent fibroblasts. Augmented expression of a number of genes involved in the TGFβ pathway suggested that TRAIL might induce TGFβ-mediated autocrine and/or paracrine stimulation of fibroblasts. This was confirmed by the ability of anti-TGFβ antibody to inhibit the effects of TRAIL on collagen production by fibroblasts. DNA mobility shift assay also revealed TRAIL-induced increase in protein binding to the collagen promoter that was substantially inhibited by consensus oligonucleotide for Smad3/4, mediators of TGFβ signaling. These data suggest that TRAIL can enhance extracellular matrix synthesis in fibroblasts by triggering TGFβ production, which acts in autocrine manner.

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Anatomic localization of dendritic cells subsets and plasmacytoid-like Th1 T lymphocytes in rheumatoid synovium: correlation with selective chemokine expression

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Rheumatoid arthritis (RA) can be considered as an ectopic lymphoid organ. This suggests a role for chemokines in the migration of T lymphocytes and dendritic cells (DCs) leading to the local organization that is characteristic of the follicular structure of RA synovium. To clarify whether DCs reach the synovium as mature cells or undergo local maturation, we characterized, by immunohistochemistry, the different DC subsets and their association with IL-17 and IFN-γ-producing T cells. The study was done in 12 RA synovium using tonsils as positive control. Immature and mature DCs were defined by expression of CD1a and DC-LAMP/CD83 respectively. Immature CD1a$^+$ DCs were mainly detected in the lining layer whereas mature DCs were exclusively detected in the perivascular or lymphocytic infiltrates. The lack of coexpression of DC-LAMP and CD1a indicated the presence of independent immature and mature DC subsets. IL-17 and IFN-γ-producing T cells were detected at the periphery of the lymphocytic infiltrates. They had a plasmacytoid-like appearance but staining for immunoglobulin light chains was negative. A similar morphotype was also observed in stimulted PBMC. To define the DC and Th1 lymphocytes migration pattern, we focused on the expression of chemokines and their associated receptors CCL20/CCR6 controlling the migration of immature DCs and activated T lymphocytes, and CCL21/CCR7, CCL19/CCR7 involved in mature DC and T lymphocyte homing. CCL20 was expressed in the lining layer and to a lower extent in the lymphocytic aggregates. CCL20 colocalized with its receptor CCR6. CCL20-producing cells were associated with CD1a+ immature DCs or IL-17-producing T cells, suggesting the contribution of CCL20 to the homing of CCR6+DC and CCR6+Th1 cells to RA synovium. CCL21 and CCL19 were only detected in the perivascular aggregates as observed for their receptor CCR7. Close association between CCL19, 21, CCR7, and mature DCs or IL-17-producing T cells argues for a role for these chemokines in the homing of CCR7+ DCs and CCR7+IL-17-producing T cells in perivascular infiltrates. The role of DCs and T lymphocytes in disease initiation and perpetuation makes the chemokines, controlling their migration, a potential therapeutic target.

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IL-18 correlates positively with CRP and Il-1Ra but not with DAS28 or bone erosion in patients with rheumatoid arthritis

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We conducted a study testing the hypothesis that IL-18 predicts disease activity in patients with rheumatoid arthritis (RA), as measured by the number of swollen joints (40 joint counts), DAS28, the sedimentation rate and bone erosion. The study consisted of 57 patients with RA and 20 control subjects. The mean age of the patients was 51 years (range 29 to 75 years). The duration of the disease averaged 7.3 years (range 0 to 30 years). The numbers (means) of swollen and tender joints were 5.2 and 5.5 respectively. The mean activity disease score (DAS28) averaged 4.9 (range 0.6 to 11). Methotrexate and systemic glucocorticoids were administered to 40% and 30% of the patients, respectively. IL-18 was significantly increased in RA patients as compared to controls. The plasma concentration of IL-18 averaged 231 and 144 pg/ml in RA patients and controls ($P < 0.001$). DAS28, CRP, sedimentation rate and IL-18 positively correlated with the plasma IL-1Ra concentration ($P < 0.001$, $P < 0.001$, $P < 0.001$ and $P < 0.002$), respectively. Stepwise backward and multiple regression analyses demonstrated that the plasma concentration of IL-1Ra correlated positively with sedimentation rate, serum triglycerides and bone erosion (r=0.74; $P < 0.001$). Plasma IL-18 correlated positively with CRP (r=0.36; $P < 0.007$) and IL-1Ra (r=0.42; $P < 0.002$) but not with the overall disease activity (DAS 28) or bone erosions. S-triglycerides correlated positively with IL-1Ra and IL-18 ($P < 0.002$, $P < 0.05$), respectively. Although IL-18 correlated positively with CRP and IL-1Ra in RA, we were unable to demonstrate any significant correlation with clinical disease score in terms of DAS28, disease duration or bone erosion.

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Adenoviral transfer of murine Oncostatin M induces inflammation and bone apposition in joints of IL-1, IL-6 and TNF-α deficient mice

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The IL-6 family member Oncostatin M (OSM) is expressed in the joints of rheumatoid arthritis patients. Murine OSM was expressed in the joints of naive wild-type mice by an adenoviral vector (AdmuOSM). This induced inflammation. Gene expression for IL-1, IL-6 and TNF-α were greatly enhanced in the inflamed synovium. To determine the role of these cytokines in the AdmuOSM induced inflammation, we injected this vector in IL-1, IL-6 or TNF-α knock-out (ko) mice. Both IL-1 and TNF-α ko mice showed reduced acute inflammation. Inflammation at day 14, however, was not reduced compared to wild-type mice. AdmuOSM induced inflammation in IL-6 ko mice did not differ from wild-type inflammation. In all the mice examined, cartilage proteoglycan depletion occurred. Depletion of proteoglycans not only occurred in the articular, but also in the epiphyseal cartilage. Early during the inflammation, the periosteum became activated and new bone apposition took place along the femur and tibia. These results suggest an important and independent role for OSM in joint pathology. Furthermore, they show that overexpression leads to damage to the epiphysis and bone apposition.

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Effect of IL-18 on synovial cell subsets and its regulation by IL-18BP

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IL-18 has multiple biological activities that are important in generating Th1 responses and inflammatory tissue damage. IL-18 has molecular similarity with IL-1, and IL-18 receptor (IL-18R) belongs to the superfamily of IL-1 receptor. In our previous studies, IL-18 was detected in both synovial fluid and serum samples from patients with rheumatoid arthritis (RA) and serum IL-18 levels correlated with disease activity as assessed by levels of serum CRP and erythrocyte sedimentation rate. We then examined the synovial expression of IL-18 in RA. Isolated RA tissue cells could spontaneously produce high amounts of IL-18 in culture. In RA synovium, IL-18-positive cells were frequently located in both the lining and sublining layer, but not in lymphocyte aggregates. Both $CD14^+$ macrophages and synoviocytes isolated from RA synovium expressed detectable levels of IL-18 mRNA. Using IL-1 as a positive control, we next examined the effect of IL-18 on IL-6 production in RA synoviocyte culture. In contrast to IL-1, IL-18 has no effect on IL-6 production from RA synoviocyte at both mRNA and protein levels. This lack of response appears to be related to defective receptor expression. Isolated RA tissue cells as well as nonstimulated RA synoviocytes expressed detectable levels of mRNA of IL-18R alpha chain, but IL-18R beta chain, which is critical for IL-18 signal transduction, was detected in RA tissue cells but not in purified synoviocytes, even if these cells were stimulated with IL-1β, TNF-α, or supernatant of RA synovium culture. RA synovial cells responded to IL-18. IFN-γ production by IL-12-stimulated RA synovial tissue cells was synergistically enhanced by IL-18, and it was partially inhibited by addition of IL-18 binding protein (IL-18BP). Upregulation of IL-18R beta mRNA levels in RA synovial tissue cells with IL-12 stimulation could explain this synergy. These results indicate a complex effect of IL-18 on various cell subsets found in RA synovium. IL-18 appears to further enhance the Th1 promoting activity of IL-12, an effect which is regulated by IL-18BP.

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The expression of IFN-β in synovial tissue from rheumatoid arthritis patients compared to osteoarthritis and reactive arthritis patients

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Objective: IFN-β may have anti-inflammatory effects in rheumatoid arthritis (RA) patients through inhibition of the production of proinflammatory cytokines like TNF-α. Increased IFN-β production in RA synovium could represent a reactive attempt to inhibit the inflammatory cascade. The aim of this study was to determine the expression of IFN-β in synovial tissue of patients with RA, osteoarthritis (OA), and reactive arthritis (ReA).
Methods: Synovial biopsy specimens were obtained by needle arthroscopy from 15 RA patients, 10 patients with inflammatory osteoarthritis (OA), and 5 patients with reactive arthritis (ReA). Immunohistologic analysis was performed using a monoclonal antibody specific for IFN-β (PBL). Bound antibody was detected according to a 3-step immunoperoxidase method. Stained sections were evaluated by computer-assisted image analysis.
Results: IFN-β was abundantly expressed in the synovial tissue of RA patients, especially by fibroblast-like synoviocytes. Digital image analysis using a standardized program revealed a statistically significant increase in the mean integrated optical density for IFN-β expression in RA synovial tissue compared with controls (RA 1900±514 vs. OA 447±171, and ReA 358±195) ($P < 0.04$). The specific upregulation of IFN-β expression was also observed when the results were controlled for cell numbers ($P < 0.02$).
Conclusions: The increased expression of IFN-β in RA synovium suggests activation of an immunomodulatory mechanism that could inhibit synovial inflammation.

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BiP may have an immunoregulatory function mediated through IL-10 production

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Aim: We have recently described BiP, a ubiquitous chaperone protein and member of the heat shock protein 70 family, as an autoantigen in rheumatoid arthritis (RA). Animal studies showed that pretreatment with BiP conferred protection from adjuvant arthritis in rats and collagen induced arthritis in mice.
Methods: Peripheral blood mononuclear cells (PBMC) were stimulated with recombinant human (rhu) BiP, rhu β-galactosidase (β-gal) and lipopolysaccharide (LPS) in the presence or absence of polymyxin B (polyB). Supernatants were collected after 24 hours and IL-10, TNF-α, IL-1β were measured by ELISA. Proliferation was measured by uptake of tritiated thymidine following stimulation of PBMC by tuberculin PPD in the presence and absence of BiP at 5 days.
Results: BiP induced production of IL-10 was significantly higher than that induced by β-gal ($P = 0.008$) and LPS ($P = 0.033$). Polymyxin B caused no inhibition of cytokine release following rhu BiP stimulation (-polyB: 4.8±1.5 ng/ml vs. +polyB: 4.6±1.6 ng/ml) although IL-10 secretion by rhu β-gal (-polyB: 4.3±1.1 ng/ml vs. +polyB: 1.7±0.4 ng/ml) and LPS (-poly B: 3.2±0.8 ng/ml vs. +polyB: 1.6±0.7 ng/ml) was significantly reduced. BiP stimulation

induced TNF-α production at a lower concentration than IL-10 and none was detectable 96 hours post-stimulation. The proliferative response of PBMC to tuberculin PPD was significantly reduced by the presence of BiP ($P = 0.0128$).
Conclusions: The increased production of IL-10 induced by rhu BiP stimulation was not due to LPS contamination of the recombinant protein. BiP may have an immunoregulatory role mediated through the production of IL-10.

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The human chaperone BiP stimulates interleukin(IL)-10 producing CD8 T cells: implications for rheumatoid arthritis

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Introduction: Rheumatoid Arthritis (RA) is the most common, crippling autoimmune disease, affecting between 0.3 and 3% of the population. Our laboratory has implicated the human chaperone, BiP, in the disease process.
Aims: This study was aimed at dissecting the T cell response to BiP.
Methods: T cell clones from normal individuals shown to respond to BiP were generated. The specificity of the clones was determined and the clonality determined by staining for the Vβ region of the T cell receptor. Supernatants were taken from the clones and control cells after stimulation with phytohaemagglutinin and cytokine production determined by ELISA. Additional phenotypic investigation was performed by flow cytometry.
Results: Out of the 6 clones isolated, which responded to BiP, 5 expressed CD8 and 1 CD4. Three clones, all $CD8^+$, grew strongly and were investigated further. T cell receptor usage was determined in two clones (Vβ 7.1 and Vβ 12) with the Vβ element of the remaining clone not being recognised by the panel of antibodies used. All three clones produced IL-10 (80–380 pg/ml), with two producing IL-4 (10–80 pg/ml) and IL-5 (>5000 pg/ml). One clone produced both IL-10 and IFNγ (>5000 pg/ml). Additional phenotyping of these clones showed them to express CD25, CD28, CD80 and 86 but not CD56 or 57. One clone constitutively expressed CTLA-4 cytoplasmically.
Conclusions: This study shows that a population of $CD8^+$ T cells, with the cytokine profile of Tc2 cells, can be stimulated by the chaperone BiP. These cells may perform a regulatory role in the normal response to inflammation. The increase in response to this antigen in the synovial joint in RA may indicate an attempt to regulate the ongoing inflammation.

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The effects of interferon-β (IFN-β) treatment on synovial cytokine expression in collagen induced arthritis in DBA/1 mice

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Objective: IFN-β is believed to have immunomodulatory properties which might have a beneficial effect in rheumatoid arthritis (RA) by inhibition of TNF-α and enhancement of IL-10 and IL-1RA production, as has been shown *in vitro*. The aim of this study was to evaluate the effect of IFN-β treatment on cytokine expression *in vivo* in an animal model of RA.
Methods: DBA/1 male mice with collagen induced arthritis (CIA) were treated with daily intraperitoneal injections of IFN-β (2.5 mg/day, 1.25 mg/day, 0.25 mg/day), or NaCL for 7 days (n=8–10). Treatment started on day 1, which represents the first day that clinical arthritis was detected in that mouse. The mice were sacrificed on day 8. Immunohistologic staining was performed on decalcified wax-embedded paw sections with polyclonal antibodies specific for TNF-α, IL-1β, IL-6, IL-10, and IL-18. The sections were evaluated by semiquantative analysis on a 5-point scale (0–4).
Results: There was a statistically significant decrease in the mean scores for expression of TNF-α and IL-6 in animals treated with the highest dose of IFN-β (2.5 mg/day) compared with the NaCL control group (TNF-α: NaCL: 2.9±0.3 vs. IFN-β 1.4±0.2) (IL-6: NaCL: 2.3±0.3 vs. IFN-β 0.9±0.3) (TNF-α: $P < 0.05$, IL-6: $P < 0.05$). IL-18 and IL-1β expression tended to be lower in IFN-β treated animals, but this difference did not reach statistical significance. Interestingly, IL-10 production was increased in the IFN-β treated animals (NaCL: 0.5±0.3 vs. IFN-β: 1.6±0.5), although not statistically significant.
Conclusion: The reduced expression of TNF-α, IL-6, IL-1β, and IL-18 and the increased expression of IL-10 in mice with CIA treated with IFN-β extends previous *in vitro* observations. Because of its immunomodulatory effects, IFN-β is a potential therapy for RA.

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Production of interleukin (IL)-1 receptor antagonist by human articular chondrocytes

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IL-1 receptor antagonist (IL-1Ra) is a member of the IL-1 family, which acts as a natural IL-1 inhibitor. The balance between IL-1 and IL-1Ra plays an important role in the course of various inflammatory diseases, such as arthritis. IL-1Ra is produced as different isoforms, one secreted (sIL-1Ra) and three intracellular (icIL-1Ra1, 2, 3), derived from the same gene. We examined the production of IL-1Ra isoforms by cultured human articular chondrocytes in response to various cytokines. The concentrations of IL-1Ra were measured by ELISA in conditioned media and cell lysates. The levels of IL-1Ra were undetectable in culture supernatants of untreated cells, but were significantly increased by IL-1β (IL-1). IL-1Ra secretion was first detectable after 24 hours and increased progressively thereafter, for at least 72 hours. In contrast, the cell lysates contained very low levels of IL-1Ra, even in response to IL-1 stimulation, suggesting that articular chondrocytes produce essentially the sIL-1Ra isoform. IL-6, which had no effect on its own, enhanced the effect of IL-1, while dexamethasone (dex) prevented the response. By RT-PCR, we observed that IL-1 and IL-6 induced mainly the production of sIL-1Ra mRNA. Furthermore, IL-1 alone or combined with IL-6 increased the levels of nascent unspliced sIL-1Ra mRNA, suggesting that sIL-1Ra expression is regulated at the transcriptional level. Consistently, reporter gene assays performed in immortalized human chondrocytes, C20/A4, showed increased sIL-1Ra promoter activity in response to IL-1 and IL-6. In conclusion, human articular chondrocytes produce sIL-1Ra in response to IL-1. This effect, which is enhanced by IL-6 and inhibited by dex, reflects increased transcription from the sIL-1Ra promoter. The production of sIL-1Ra by chondrocytes may have a protective effect against articular inflammatory and catabolic responses.

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Loss of surface CXCR3 expression in the RA synovial CD3 cells as a result of ligand binding suggests the mechanism for increased Th1 cell infiltration

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Introduction: Inflammatory cell infiltration in synovium is a characteristic feature of rheumatoid arthritis (RA). Chemotactic gradients of various chemokines are responsible for cell attraction and possibly for their activation. Th1 cells are the predominant T cells in the synovium and have been shown to express high levels of chemokine receptors CXCR3 and CCR5.
Aim: To examine a role of CXCR3 and its respective ligands (IP-10 and MIG) in the migration of Th1 cells into the synovial tissue.
Methods: Synovial tissue samples were obtained from 12 RA patients undergoing either synovectomy or a total joint replacement. Cells were released by digestion with collagenase, DNAse and briefly with hyaluronidase. A three-colour fluorescence analysis was performed with FITC conjugated anti-CXCR3 MAb (R&D) and with PE conjugated anti-CD3. Live cells were identified by propidium iodide. PBCs were stained using the same protocol. Cells were permeabilized with IntraPrep for intracellular staining. Cryostat tissue sections were stained for CXCR3 ligands IP-10 and MIG.
Results: In the investigated RA patients 18–70% of PB $CD3^+$ cells expressed surface CXCR3. $CD3^+$ population of cells in the synovium expressed 0–5% of surface CXCR3. The intracellular CXCR3 was found in 97–100% of synovial $CD3^+$ cells. IP-10 and MIG were expressed in all tissue samples. This staining was particularly enhanced in tissues with strong vascularity. Endothelial cells were strongly positive for IP-10 and weakly positive for MIG.
Conclusions: The intracellular presence of CXCR3 and simultaneous loss of surface expression in the synovial $CD3^+$ cells is the result of internalization of this molecule upon ligand binding. The presence of IP-10 and MIG in the synovium suggests the possible method of Th1 cell migration and their further activation.
Acknowledgement: This work was supported by a grant NI/6459 from IGA MZ CR.

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Differential influence of IL-1-β, TNF-α, and PDGF-BB on the expression of matrix-metalloproteinases (MMPs) and 'total'-MMP activity in early-passage RA- and OA-synovial fibroblasts (SFB)

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Objectives: To characterize the influence of stimulation with IL-1-β, TNF-α or PDGF-BB on the mRNA expression of MMP-1 to MMP-19 in rheumatoid arthritis (RA)-SFB and osteoarthritis (OA)-SFB, and to assess the 'total' MMP activity in the supernatants of the cells.
Methods: RNA was isolated from 2nd passage RA- and OA-SFB (enrichment 98%; with/without stimulation by IL-1-β, TNF-α or PDGF-BB for 24 hours). The expression of MMP-1 to MMP-19 was analyzed by semiquantitative RT-PCR with gene-specific primers. 'Total' MMP-activity was assessed in the supernatant of stimulated RA- and OA-SFB using the nonspecific fluorogenic MMP-substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2.
Results: No significant differences were observed between unstimulated 2nd passage RA- and OA-SFB for the expression of any MMP. Following IL-1-β stimulation, MMP-1, MMP-2, MMP-3, MMP-9, MMP-10, MMP-13, and MMP-19 were upregulated in RA-SFB, but only MMP-3, MMP-9, MMP-10, and MMP-12 in OA-SFB. In addition, MMP-11 and MMP-14 were downregulated in OA-SFB. TNF-α upregulated MMP-1, MMP-9, and MMP-13, but downregulated MMP-8, MMP-12, MMP-14, and MMP-19 in RA-SFB. In OA-SFB, TNF-α upregulated MMP-3, MMP-7, and MMP-9, but downregulated MMP-8 and MMP-16. Following PDGF-BB stimulation, MMP-8 was upregulated, but MMP-3, MMP-10, and MMP-13 were downregulated in RA-SFB. Strikingly, stimulation of OA-SFB with PDGF-BB led to significant downregulation of 8 MMPs (MMP-1, MMP-2, MMP-3, MMP-8, MMP-11, MMP-12, MMP-13, and MMP-14). IL-1-β and TNF-α significantly downregulated 'total' MMP-activity in the supernatants of OA-SFB, but not in the supernatants of RA-SFB. In contrast, PDGF-BB downregulated 'total' MMP-activity in the supernatants of both RA-SFB and OA-SFB.
Conclusion: IL-1-β and TNF-α may contribute to prodestructive features of RA-SFB by upregulating MMP expression and by failing to downregulate 'total' MMP activity. More extensive downregulation of MMP expression in OA-SFB than in RA-SFB suggests a differential, tissue-protective role of PDGF-BB in different inflammatory joint diseases.

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Heterogeneity in cytokine profile after intra-articular steroid treatment

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Introduction: It is well known that intra-articular steroids are efficient and fast in reducing arthritis symptoms. However, treatment does not exclude long-term morbidity. Earlier studies have shown individual patterns in cytokine expression both inherently and in response to treatment. In this study we investigated the effect of ia steroids on the synovial tissue before and 10 days after treatment.
Patients and methods: Serial arthroscopical synovial biopsies were taken from patients with knee arthritis, before and ten days after ia triamcinolone injection. Biopsies were stained for histology, cytokines, cellmarkers and adhesion molecules using monoclonal antibodies, and measured by digital analysis or conventional counting of cells. Statistics were performed by Wilcoxon paired *t*-test.
Results: Almost all patients (10/11) improved clinically and macroscopically by arthroscopy. Only ICAM-1 and CD3 were significantly reduced. Very small amounts of TNF-producing cells were observed with a clear trend of reduction, but individual patterns were observed. No overall changes were observed for CD68/163, IL-1α or IL-1β.
Conclusion: This is the first study where the cytokine pattern has been analysed after intra-articular steroid injection and almost all patients improved clinically. However, there was no reduction in synovial macrophages, but T-cells as well as ICAM-1 diminished. No change in IL-1 was detected. TNFα producing cells were remarkably few with a trend to diminish, but individual patterns were seen. In this study, steroid treatment did effect the inflammation but it did not disappear completely microscopically as well as macroscopically. The therapeutic effects have mostly been short-lived and this study may provide an explanation for this.

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Local inhibition of endogenous IL-18 through adenoviral overexpression of IL-18BPc results in reduced incidence and severity of collagen induced arthritis in mice

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IL-18 is a member of the interleukin-1 family and plays an important role in innate and acquired immunity. Previous experiments focused on IL-18 in experimental arthritis showed that neutralization through systemic treatment with either antibodies or IL-18 binding protein (IL-18BP) ameliorated the disease. To study the local role of IL-18 in arthritis we developed a replication deficient adenoviral vector containing the murine IL-18BP isoform c gene (Ad5CMV.IL-18BPc). The neutralizing ability of adenoviral overexpressed IL-18BPc on IL-18 response was tested *in vitro*. Supernatants of IL-18BP transfected cells significantly inhibited IL-18 induced luciferase production in IL-18 sensitive NFkB luciferase reporter fibroblasts. Next we injected 1×107 PFU of Ad5CMV.IL-18BPc or the control vector Ad5CMV.Luc into the murine knee joint cavity of DBA/1 mice before onset of collagen-induced arthritis (CIA). IL-18BPc overexpression significantly reduced local knee joint swelling in CIA with 66% ($P < 0.001$) and distal paw swelling with approx. 50% ($P < 0.01$) compared to the control vector. Furthermore, local IL-18BPc resulted in lower serum levels of IL-6 compared to the control (respectively 5.0 pg/ml and 12.7 pg/ml, $P < 0.001$). Serum titers of specific IgG1, IgG2a and total IgG antibodies directed towards collagen type II showed no significant differences between control and IL-18BPc treatment, indicating that the effect seen on arthritis by overexpression of IL-18BPc in the joint was not caused by an IL-18BPc effect on humoral immunity. These results clearly show that IL-18 present in the arthritic joint plays an important proinflammatory role and demonstrate that adenoviral overexpression of IL-18BPc in the synovium is an efficacious local treatment in experimental arthritis.

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The bias for Th1 cell differentiation of rheumatoid arthritis T cells is characteristic of memory but not of naive T cells

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An impaired ability of CD4 memory T cells to differentiate into immunomodulatory Th2 cells has been documented in patients with rheumatoid arthritis (RA) and has been implicated in the initiation and perpetuation of the characteristic Th1 dominated rheumatoid inflammation. To determine the stage of T-cell maturation at which the bias for Th1 cell differentiation becomes manifest, we investigated Th cell differentiation from resting CD4 CD45RA (naive) and CD45RO (memory) T cells in patients with untreated early RA (mean disease duration <8 months) and in age- and sex-matched healthy controls *in vitro*. No differences in the cytokine secretion profile, as assessed by flow cytometry, between patients and control subjects were detected in freshly isolated naive or in freshly isolated memory T cells. Th2 cells could be induced from naive T cells in all healthy donors and in all RA patients by priming with monoclonal antibodies (mAbs) to CD3 and CD28. Exogenous IL-4 was not required for Th2 differentiation from naive cells and did not consistently increase Th2 priming efficacy. In contrast to naive cells, Th2 effectors were effectively generated from memory T cells by stimulation with anti-CD28 without ligation of the T-cell recceptor. This mode of stimulation generated Th2 cells from memory T cells of all healthy controls but in only one third of the RA patients. The data suggest that CD4 memory T cells from the majority of patients with early RA manifest a deficiency in their capacity to differentiate into Th2 effectors. In contrast, naive T cells are capabale of differentiating into Th2 cells with appropriate stimulation suggesting that the bias in Th1 differentiation of most RA patients may be acquired during T-cell maturation presumably in response to antigenic stimulation.

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Induction of Th2 cell differentiation by cognate interaction of CD4 memory T cells

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Th2 cell differentiation can be induced from human CD4 memory T cells by priming with anti-CD28 without engagement of the TCR. To test whether CD28 mediated Th2 differentiation from memory T cells was an effect of a particular anti-CD28 mAb, Th2 differentiation was assessed after priming of purified CD4 memory T cells with transfected myeloma cells expressing human CD80 and/or CD86. Whereas the mock control did not induce Th2 differentiation, priming in the presence of myeloma cells expressing CD80, CD86 or CD80 and CD86 significantly increased Th2 frequencies. Thus, Th2 differentiation from memory T cells can be induced by engaging CD28 with its natural ligands, CD80 and/or CD86. We next evaluated the possibility that activated T cells might prime bystander T cells for Th2 differentiation. CD4 memory T cells were activated with mAbs to CD3 and CD28 for five days. Flow cytometric analysis revealed that in contrast to resting T cells, activated T cells expressed CD80, however, at a low level. CD80 expressing T cells were fixed and cocultured with freshly isolated, syngeneic, CD4 memory T cells for five days in the presence or absence of exogenous IL-4, but without additional stimulatory signals. For control, priming was performed with fixed syngeneic T cells that were freshly isolated and, thus, negative for CD80. Priming with fixed, non-activated syngeneic T cells did not induce Th2 differentiation, even in the presence of exogenous IL-4. In marked contrast, priming with fixed activated T cells induced significant Th2 differentiation. This effect was dependent on exogenous IL-4, suggesting that the low levels of CD80 expressed on activated T cells were not sufficient to induce IL-4 transcription. However, the data indicate, that Th2 differentiation might be induced from bystander T cells by recently activated T cells through cognate T–T-cell interaction involving CD28. TCR independent generation of Th2 effectors might provide a way to control Th1 dominated cellular inflammation.

Autoantigens and autoantibodies

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Comparison of different immunoassays detecting anti-chromatin autoantibodies in systemic lupus erythematosus (SLE)

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Introduction: Anti-double-strand DNA (dsDNA) autoantibodies are considered a hallmark of SLE and many assays have been devel-

oped to improve the determination of these antibodies. Recently, autoantibodies to nucleosome, the fundamental unit of chromatin, have been shown to constitute a new and very specific marker of SLE.

Methods: To evaluate the diagnostic value of (A) Crithidia lucillae indirect immunofluorescence (CLIFT), anti-dsDNA immunoassays using (B) purified dsDNA (Quanta LiteTM dsDNA ELISA INOVA, San Diego, CA), (C) recombinant plasmid dsDNA (EliATM, Pharmacia, Freiburg, Germany) and (D) anti-nucleosome immunoassay (Quanta Lite Chromatin ELISATM, INOVA, San Diego, CA), we investigated in a prospective study all the sera sent to our laboratory for anti-dsDNA detection during 2 months. 122 sera were enrolled in this study of which 16 appear to be from SLE patients and we assessed the sensitivity and specificity of the assays. We also compared a group of 30 SLE patients with other autoimmune diseases (19 cutaneous lupus, 22 Sjögren's syndrome, 14 systemic scleroderma) in a retrospective study.

Results: In the prospective study, the sensitivity for SLE was 13%, 53%, 47% and 60% for test A, B, C and D, respectively; the specificity was 99%, 94%, 94% and 99% for test A, B, C and, respectively. Results were similar in the retrospective study.

Conclusion: Anti-nucleosome autoantibodies are sensitive and specific markers for SLE. However, other studies are necessary to evaluate if they are also useful in monitoring disease activity.

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Anti-keratin and anti-cyclic citrullinated peptide autoantibodies in patients with juvenile idiopathic arthritis

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Objective: We discuss the presence of anti-keratin antibodies (AKA) and anti-cyclic citrullinated peptide (anti-CCP) antibodies of the IgG class in sera of patients with defined juvenile idiopathic arthritis (JIA) of various subgroups with more than one year duration of the disease.

Methods: An indirect immunofluorescence test on rat oesophagus substrate (ImmuGloTM, Immco Diagnostics, Buffalo, USA) and enzyme-linked immunosorbent assay (Immunoscan RA, Eurodiagnostica, The Netherlands) were used for the detection and quantification of AKA and anti-CCP antibodies in 140 patients with JIA (64 male and 76 female) aged 2–47 years (median 16.5 years).

Results: Overall, AKA were found in 40/140 patients (28.6%, $P = 0.04$) including 2/11 systemic arthritis (18.2%), 2/32 oligoarthritis (6.3%), 18/52 RF negative polyarthritis (34.6%, $P = 0.01$), 14/18 RF positive polyarthritis (77.8%, $P = 0.000002$), 2/15 enthesitis related arthritis (13.3%) and 2/3 psoriatic arthritis patients. AKA were not found in a very small cohort of patients with extended oligoarthritis (n=4) and unclassifiable arthritis (n=5). Only 7/122 (5.7%) patients were positive for anti-CCP. The correlation between AKA and anti-CCP was 77.1% ($P = 0.05$).

Conclusion: We conclude that while AKA antibodies measured using IIF on rat oesophagus can be detected in patients with definite JIA with more than 1 year duration of the disease, only rare occurrence of anti-CCP was observed. We conclude that AKA occurred in any JIA patient category, however when statistically compared with healthy controls significantly higher frequencies were found only in a cohort of RF positive and RF negative polyarthritis.

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The effect of infliximab on rheumatoid factor and anti-citrullinated cyclic peptide antibodies

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Background: Rheumatoid arthritis (RA) patients are successfully treated with infliximab (anti-TNF-α therapy). This therapy may induce autoantibodies like anti-dsDNA antibodies.

Objectives: To investigate the effect of infliximab on rheumatoid factor (RF) and anti-citrullinated cyclic peptide antibodies (anti-CCP antibodies).

Patients and methods: 62 patients with refractory RA were treated with infliximab in an early access program. They received 3 mg/kg infliximab IV at week 0, 2, 6 and every 8 weeks thereafter in combination with MTX. Serum samples were obtained at baseline and week 30. Samples were tested for RF Waaler-Rose (SERODIA-RA, Fujirebio inc), RF Latex Fixation (DifcoLaboratories) and anti-CCP antibodies ELISA (Euro-Diagnostica).

Results: At baseline, 41/62 RA patients were positive for RF Waaler-Rose, 39/62 RA patients were positive for RF Latex Fixation and 41/62 RA patients had a positive testresult for anti-CCP antibodies ELISA. After infliximab treatment, 33/62 RA patients were positive for RF Waaler-Rose, 31/62 RA patients were positive for RF Latex Fixation and we observed a positive test result for anti-CCP antibodies in the same 41/62 RA patients. McNemar test revealed a significant paired difference for RF Waaler-Rose ($P = 0.039$) and for RF Latex Fixation ($P = 0.021$), but not for anti-CCP antibodies ($P = 1.0$). Wilcoxon Signed Rank Test showed a significant decrease for RF Waaler-Rose ($P < 0.001$) and for RF Latex Fixation ($P = 0.035$). No significant difference was observed for anti-CCP antibodies ($P = 0.744$).

Conclusion: In RA patients, infliximab treatment results in a decrease of RF Waaler-Rose and RF Latex Fixation. No difference considering the anti-CCP antibodies is seen.

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Infliximab induced anti-dsDNA: characteristics of autoantibody response

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We have previously been reported that 7–12% of patients with rheumatoid arthritis develop IgM anti-dsDNA following treatment with infliximab and that lupus was observed in 1 of 156 patients and was associated with and IgG and an IgM anti-dsDNA response (Charles *et al. Arthritis Rheum* 2000, **43**:2883-2900). In this study we examine the characteristics of the autoantibody response to find evidence for the involvement of potential mechanisms including upregulated synthesis of natural autoantibodies, polyclonal IgM activation, the production of cross reactive rheumatoid factors, or a response to apoptotic release of nucleosomal material.

Methods: IgM antibodies to IgG (rheumatoid factor), mitochondrial, microsomal, measles antigens and circulating nucleosome levels were measured prior to and following infliximab therapy. Sera containing induced anti-dsDNA antibodies were studied by immunofluorescent inhibition to examine cross reactivity.

Results:

- IgM antibodies to mitochondrial, microsomal and measles antigens were unchanged following infliximab therapy (pretreatment vs. post-8 weeks=ns).

- IgM anti-dsDNA were inhibited by dsDNA, but not by rabbit or human IgG, or histones.
- Levels of circulating nucleosomes fell following infliximab therapy (pre-treatment vs. post-2 weeks $P \leq 0.02$; vs. post-4 weeks $P \leq 0.05$).

Conclusion: We were unable to find any evidence to support a role for upregulation of IgM natural autoantibodies, a polyclonal increase in IgM antibodies, cross reactive rheumatoid factors, or nucleosome release and consequent antigen drive. The induction of anti-dsDNA antibodies appears to be dependent on an, as yet, ill understood mechanism.

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Specificity of rheumatoid synovial B-cell hybridoma with germline configurated IGVH genes against human nuclear protein histone

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B cells (plasma cells and B-lymphocytes) belong to the dominant part of the inflammatory infiltrate in RA synovitis. To disclosure the nature of antigens involved in the triggering and the perpetuation of the disease we carried out the study of IgG, a monoclonal B-cell hybridoma, belonging to the VH3 family (DP 43), from rheumatoid synovial membrane, produced by electrofusion and without prior *in vitro* stimulation. The subject of the study was an RA patient with definite and active RA, featuring dense inflammatory infiltration with the occurrence of secondary lymphatic follicles in the affected joint. This hybridoma was analysed by PCR, immunohistology, immunoprecipitation and western blot, and the results were correlated with pathological data and parameters of local disease activity. By PCR we found out that this hybridoma had germline configuration IgVH genes with R/S ratio=1; it revealed specific binding (20 kDa) to nuclear extract of fibroblasts cell line (Hep2), later identified by mass spectrometry as the nuclear protein human histone (1B, 1C, 1D). Being this protein an exclusive nuclear protein involved in the chromatin condensation, it can be assumed that its implication in RA will be related to the perpetuation of the disease and inflammation after massive cell death, due to destruction of the joint tissues.

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Immunomic analysis of syovial fluid exosomes of rheumatic diseases patients

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In addition to soluble proteins and mediators, cells also release membrane vesicles in the extracellular environment, exosomes and apototic blebs. Apoptotic blebs have been studied and contain multiple autoantigens. Exosomal protein content and its functions are starting to be unraveled. During an immune response the 40 nm particles are formed, on the surface they have Class I and II antigens as well as viral proteins or other proteins like heatshock proteins Hsp 70 and 90, or B7 a costimmulatory molecule. Recent studies showed that exosomes can stimulate T cells directly or bind to dendritic cells, suggesting a general function for exosomes in immune response. The mechanism of action *in vivo* is poorly understood. Exosomes seem to stimulate T cells and have been shown to induce tumor rejection when loaded with tumor peptide. The composition of synovial exosomes has so far not been studied or compared to other diseases and normals. Autoimmune diseases like rheumatoid arthitis (RA) are characterized by autoantibodies to different proteins like BiP, hnRNPs and p205 which has not been charaterized in its nature. Different synovial exosomes from RA patients, reactive arthritis, and osteoarthritis patients and controls have been purified and anlysed for specific autoantigenic content by immunoblotting. P205 was found in all exosomal preperations in contrast to Bip and hnRNP A2, which could not be detected in the synovial exosomes fractions so far. Synovial Exosomal proteins haved been analysed by immunoblotting after 2D electrophoresis and immuneprecipitation. Based on that methods the specific autoantigenic protein content can be analysed now. After revealing the primary structure and molecular identity of the autoantigens, recombinant autoantigens will be produced and used for diagnostic as well as immunemodulatory purposes. In summary, the autoantigenic analysis of synovial exosomes revealed that p205 is associated with exosomal particles. The investigation of the synovial exosome protein content as well as the analysis of B and T-cell sitmulation potential, may thereby possibly lead to novel diagnostic and therapeutic strategies in rheumatic diseases.

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Protein microarray characterization of autoantibody responses in rheumatoid arthritis and systemic lupus erythematosus

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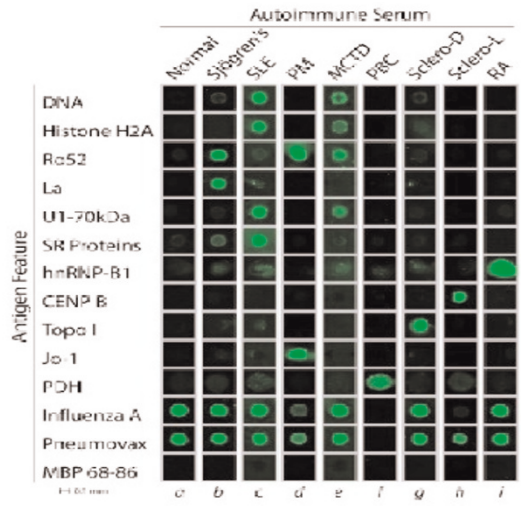
In rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and other autoimmune rheumatic diseases, our understanding of the specificity of the autoantibody response and the role of autoantibodies in pathogenesis are limited. We developed antigen microarray technology to perform multiplex characterization of autoantibody responses. Antigen microarrays are produced by attaching thousands of proteins and peptides to addressable locations on the surface of solid supports using a robotic arrayer. Arrays are probed with serum from disease and control patients, followed by anti-human secondary antibodies covalently-conjugated to spectrally-resolvable fluorochromes. We initially developed a 'connective tissue disease' array containing structurally diverse autoantigens including nucleic acids, histones, hnRNPs, snRNPs, collagens, Ro, La, SCL-70, CENP-B, Jo-1, pyruvate dehydrogenase, and post-translationally-modified antigens. Array analysis of serum derived from patients with SLE, rheumatoid arthritis, Sjögren's syndrome, mixed connective tissue diseases, scleroderma, myositidies, and primary biliary cirrhosis identified autoantibody response patterns characteristic of these diseases (Fig. 1). We are now developing 'synovial proteome' arrays to study the autoantibody responses in RA, and have produced first-generation arrays containing 450 distinct protein and peptide candidate antigens derived from synovial joints. These include native and cit-

Figure 1



rulline-modified filaggrin peptides, native and deiminated fibrinogen and vimentin, glucose-6-phosphate isomerase, collagen types I, II, III, IV and V, hnRNP-A2/RA33, hnRNP-D, immunoglobulins, HCgp39, BiP, and HSPs 60, 65, 70, and 90. We probe our arrays with sera and synovial fluid from RA and control patients. We are using our arrays to examine serial serum samples from patients with early RA to: (1) identify antigens targeted early in disease, and (2) examine for evidence of B-cell epitope spreading. 'Synovial proteome' arrays represent a powerful tool to study the breadth and specificity of autoreactive B-cell responses, and to identify candidate and define relevant autoantigens in RA.

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Do IgA antiphospholipid antibodies have any clinical importance?

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Antiphospholipid antibodies (aPL) are strongly associated with clinical manifestations of the antiphospholipid syndrome (APS) such as thromboembolic events and/or pregnancy loss. IgG anticardiolipin (aCL) at moderate/high titre and IgG anti-β2-glycoprotein I (aB2GPI) are closely related to clinical complications, IgM are commonly transient, present during infections, while IgA are not routinely tested. The aim of our study was to evaluate the occurence and possible clinical significance of the IgA isotype of four different aPL: aCL, aB2GPI, anti-prothrombin (aPT) and anti-annexin V (aANXV) antibodies. Sera from 92 patients (87 females and 5 males) with systemic autoimmune disorders (63 with systemic lupus erythematosus (SLE), 19 with secondary APS (SLE with APS) and 10 with primary APS (pAPS)) were assayed with four different in-house ELISA tests. For each of the four antibodies IgG, IgM and IgA isotypes were determined. In evaluating the association of isotypes with particular clinical features (arterial and venous thromboses, CNS disorders, abortuses, thrombocytopenia), IgA isotype did not improve the clinical sensitivity of any measured antibody. The IgA aB2GPI were the only aPL occuring alone in a significant number of patients. Such patients should be followed-up to provide insight into the possible clinical significance of IgA aB2GPI.

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Cell-free Fc-γ receptors IIIb, autoantibodies and related immune complexes trigger the production of G-CSF and GM-CSF in nonorgan-specific autoimmune diseases

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The FcγIIIb and IIa classes of receptors for the Fc portion of IgG are constitutively expressed in polymorphonuclear neutrophiles (PMN). High levels of cell-free (CF) FcγRIIIb were detected in nonorgan-specific autoimmune diseases, as well as related autoantibodies (Ab) present in 56, 58 and 51% of the patients with primary Sjögren's syndrome, systemic lupus erythematosus and rheumatoid arthritis, respectively. These autoAb were categorized, based on the results of an indirect immunofluorescence (IIF) test for the detection of anti-membrane-bound FcγRIIIb Ab, and an enzyme-linked immunosorbent assay for that of Abs recognizing only CF FcγRIIIb. Those sera positive in the IIF test were not cytotoxic ; rather, they enhanced the survival of PMNs (5 methods). The autoAb-triggered antiapoptotic signal was transduced from FcγRIIIb, either directly or through FcγRIIa and/or CD11b, (the β-chain of the neighboring complement receptor type 3). CF FcγRIIIb produced similar effects, as well as immune complexes made up of CF FcγRIIIb and anti-CF FcγRIIIb auto Abs. Anti-FcγRIIIb autoAb-conditioned supernatant had the capacity to induce the transcription of messenger RNA for G- and GM-CSF, resulting in the synthesis of these antiapoptotic factors. Such a delay in apoptosis was associated with a downregulated expression of Bax, whereas Bcl-2 was unmodified. Differed apoptosis of PMNs should occur in patients with anti-FcγRIIIb autoAbs, particularly in the rheumatoid synovial fluid. Treatment with anti-G- and/or anti-GM-CSF might thus be proposed.

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Cerebrovascular insult and antiphospholipid antibodies in young adults

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Antiphospholipid antibodies (aPL) are strongly associated with arterial and/or venous thrombosis and recurrent foetal loss. The only neurological manifestation satisfying the diagnostic criteria for the antiphospholipid syndrome (APS) is ischemic cerebrovascular insult (CVI). Because of previous contradicting reports on the association between aPL and CVI, we examined the occurrence of different aPL in a group of patients with CVI without an evident systemic autoimmune disease. 39 patients (26 women, 13 men, all under 40 years) were included in study. Blood withdrawal was performed twice after CVI at least 8 weeks apart. Sera were tested by enzyme linked immunosorbent assays (ELISA) for the presence of IgG, IgM and IgA anti-cardiolipin (aCL), anti-β2-glycoprotein I (aβ2GPI), anti-prothrombin (aPT) and anti-annexin V (aANXV) antibodies. Increased levels of IgG aCL were found in 3 (8%) patients and for IgA aβ2GPI in 1 (3%). In all aCL and aβ2GPI positive CVI patients only low positive levels of antibodies were detected. aPT were completely absent while aANXV were detected in 4 (10%) patients (3 had IgG and one IgM). aPL directed against serum antigens did not appear with a significant frequency in the studied group of CVI patients. But aANXV, directed against a tissue protein, might be important as one of the possible markers in at least some patients with CVI.

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Interference of PR3-ANCA with the enzymatic activity of PR3

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Introduction: Anti-neutrophil cytoplasmic antibodies (ANCA) against proteinase 3 (PR3) are strongly associated with Wegener's granulomatosis (WG) and are thought to be involved in its pathogenesis. *In vitro* functional effects of these antibodies have been suggested to correspond better to disease activity than levels of PR3-ANCA.
Methods: To investigate the relation between functional effects of PR3-ANCA and disease activity, we tested IgG from sera of 43 WG patients and four controls for their capacity to interfere with the proteolytic activity of PR3. Blood was drawn either during active disease or during remission of WG. The enzymatic activity of PR3 was determined using MeSuc-AAPV-pNA, casein, and by complexation of PR3 with α-1-antitrypsin (α-1-AT).
Results: Most of the IgG samples from WG patients inhibited the enzymatic activity of PR3 and the complexation of PR3 with α-1-AT. A difference in the capacity to interfere with proteolysis of casein and with complexation of PR3 with α-1-AT was observed between samples taken during active disease and during remission of WG, but this was not observed for the hydrolysis of MeSuc-AAPV-pNA. However, PR3-ANCA titers giving fifty percent inhibition of the PR3/α-1-AT complexation and the proteolytic activity of PR3 for the hydrolysis of MeSuc-AAPV-pNA were lower for remission samples compared to samples during active disease, indicating a relatively higher inhibitory activity in the former samples. PR3-ANCA titers correlated with the inhibitory activity both for patients with active disease and for patients during remission.
Conclusion: With a fixed amount of IgG, PR3-ANCA-containing IgG from patients with active disease had a higher inhibitory capacity towards the proteolytic activity of PR3 than did PR3-ANCA-containing IgG from patients during remission of WG. However, when correcting the results for the PR3-ANCA titer, PR3-ANCA of patients during remission had a relatively higher inhibitory capacity towards the proteolytic activity of PR3 than did PR3-ANCA of patients during an active phase. These results may indicate that PR3-ANCA of patients with active disease recognize different epitopes on PR3 than do PR3-ANCA of patients during remission of WG. These findings may have relevance for the pathogenicity of the antibodies.

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Anti-Ro/SSA antibodies in systemic sclerosis (SSc): determination of the fine specificity, clinical and laboratory correlations

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Background: The frequency of anti-Ro/SSA in SSc varies from 3% to 11% with immunoprecipitation assays and from 12% to 37% with ELISA. Significant associations were reported between anti-Ro/SSA and Sjögren's syndrome, pulmonary involvement and myositis in patients with SSc.
Objective: To evaluate the frequency the association of anti-Ro/SSA antibodies and their fine specificity with clinical and immunological features in SSc.
Patients and methods: We studied 193 patients with SSc (58 diffuse, 129 limited and 6 overlap syndromes) attending our outpatient clinic. Antibodies to ENA were determined by counter-immunoelectrophoresis (CIE) using rabbit thymus (Peel-Freeze) and human spleen extract as substrates. ELISA assay with recombinant 52 and 60 kD Ro proteins (Pharmacia) was performed on 107 sera, cut-off values were determined testing 75 sera from routine: 50 ANA negative and 25 ANA positive-ENA negative.
Results: Anti-Ro was detected by CIE in 12/193 patients (6%) and by ELISA in 14/107 (13%). As a whole, CIE and/or ELISA detected the presence of anti-Ro in 20 patients (Ro+). It was significantly associated with photosensitivity ($P = 0.02$), xerophtalmia ($P = 0.05$), raised ESR ($P = 0.02$), hyperγglobulinemia ($P = 0.002$), anti-La ($P = 0.004$) and antinucleolar antibodies ($P = 0.02$). Anti-Scl70 antibodies tended to be specifically associated with anti-Ro60 as compared with anti-Ro52 antibodies ($P = 0.06$). Anti-Ro+ patients had higher incidence of interstitial lung disease and slight reduction of DLCO at the moment of first evaluation, as compared with patients Ro– ($P = 0.06$), particularly with the subset anti-centromere positive ($P = 0.001$). However, decrease of DLCO during follow-up was more frequent in patients Ro– ($P < 0.001$). This observation might be partially related to a longer steroid treatment received by patients Ro+ ($P < 0.03$).
Conclusion: Antibodies to Ro/SSA are not infrequently detected in SSc. They are associated with a subset of disease characterized by clinical and serological features commonly associated with anti-Ro/SSA independently from the underlying disease. Anti-Ro+ SSc patients tended to have an earlier lung involvement apparently less evolving as compared to anti-Ro– patients.

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Autoantibodies to the TNF-α translational regulators TIA-1 and TIAR occur frequently in sera of patients with SLE and scleroderma

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Objective: The two closely related RNA binding proteins TIA-1 and TIAR are involved in post-transcriptional regulation of TNF expression by acting as translational silencers via association with the TNF mRNA. Given that TNF is upregulated in many inflammatory and autoimmune conditions it was the aim of this study to investigate the possible occurrence of autoantibodies (aab) against TIA-1 and TIAR in patients with rheumatic diseases.
Methods: Recombinant proteins were employed in immunoblotting studies involving sera of 40 patients with SLE and 20 sera each of patients with systemic sclerosis (SCL), poly/dermatomyositis (PM/DM), Sjögren's syndrome (SS), rheumatoid arthritis (RA), reactive arthritis (REA) as well as sera of 20 healthy controls (HC).
Results: IgG aab against TIA-1 and/or TIAR were frequently detected in sera of patients with SLE or SCL: thus, 67% of SLE sera (anti-TIA1: 52%, anti-TIAR: 47%) and 50% of the SCL sera (anti-TIA1: 40%, anti-TIAR: 40%) contained either of the two aab. These aab were detected at lower frequency also in sera of patients with PM/DM (30%), REA (25%), SS (15%) and RA (10%). However, only PM/DM patients differed significantly ($P < 0.05$) from healthy controls (5%). Due to the close structural homology between the two proteins the majority of sera were reactive with both of them suggesting a high degree of crossreactivity. Nevertheless, some sera contained autoantibodies directed specifically to only one of two antigens.

Conclusion: Autoantibodies to TIA-1 and TIAR occurred frequently in patients with SLE and SCL but, interestingly, were rarely detectable in patients with RA. Thus, these data suggest that in susceptible individuals systemic upregulation of TNF may lead to autoimmune reactions against proteins associated with the TNF mRNA.

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Molecular cloning of candidate antigens recognized by anti-endothelial cell antibodies

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Anti-endothelial cell antibodies (AECA) have been reported in such a vast array of conditions associated with vasculitis that the related autoantigens have become extremely difficult to identify. We have, therefore, used a molecular cloning approach to define endothelial cell (EC) target structures by screening a human umbilical vein EC (HUVEC) cDNA expression library. Total RNA was extracted from HUVECs and purified by oligo-dT cellulose chromatography. A commercial kit was used to synthesize cDNA which was inserted into the *EcoRI/XhoI*-digested and dephosphorylated lambda ZAP Express vector. The construct was incorporated into phages and transformed with *E. coli* strain XL1 Blue. After the addition of AECAs, positive clones were isolated in suspending medium, purified and incorporated again into the XL1. The insert-containing phagemids were exised from recombinant phages and introduced into *E. coli* XLOLR strain. The restriction fragments, obtained from the cDNA inserts by *EcoRI* endonuclease digestion, were sequenced by the dideoxy-chain termination method with two primers, T3 (AATTAACCCTCACTAAAGGG) and T7 (CGGGATATCACTCAGCATAATG). The sequences were examined for analogy in the GenBank and EBML nucleic acid data banks. Two sera, one from a patient with systemic lupus erythematosus (SLE) and another from a patient with Wegener's granulomatosis (WG), both strongly AECA-positive in our in-house cell-ELISA, were used to screen the bank. The SLE serum recognized methyltransferase, NADH dehydrogenase and unidentified components, whereas the WG serum recognized methyltransferase, GAPDH and unidentified structures. AECAs could thus be associated with a bulk of autoantibodies targeting various enzymes, or get inside the ECs to bind to such proteins.

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Sensitivity of ANA indirect immunofluorescence testing for the detection of anti-ENA antibodies

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Background: Detection of antinuclear antibodies (ANA) is mostly performed by indirect immunofluorescence (IIF) on HEp2 cells. The HEp2000 substrate, consisting of HEp2 cells transfected with Ro60 cDNA, could be a valuable alternative, with increased sensitivity for detecting anti-SSA antibodies. When IIF is positive, further determination of the fine specificities is performed. However, some reactivities can be missed on IIF (anti-SSA, anti-Jo1).
Objectives: 1) To compare the performance of the HEp2 and HEp2000 substrate for ANA detection. 2) To determine the sensitivity of ANA IIF testing for the detection of anti-ENA antibodies.
Patients and methods: 495 samples consecutively referred for routine ANA testing by rheumatologists were collected. IIF on HEp2 and HEp2000 and Line Immunoassay (INNO-LIA ANA, Innogenetics, Belgium) was performed on all samples.
Results: Fluorescence intensities and patterns on HEp2 and HEp2000 were strongly correlated (Spearman's Rho=0.838; $P < 0.001$ and 0.787; $P < 0.001$, respectively). 292 samples were negative on HEp2 and HEp2000, 18 of these had a positive LIA result. Reactivities seen are towards one or more RNP antigens (8 patients); Ro52 and Ro60 (1 patient); mono SSB (4 patients); Jo1 (1 patient); SmD (1 patient); RNPC, Ro52, Ro60, SSB, sclero70 (1 patient); RNPC and SSB (1 patient); SmB (1 patient). Two out of 26 patients, positive on HEp2 but negative on HEp2000, showed reactivities: one with anti-Jo1 and one with anti-RNPC. Three out of 23 patients, positive on HEp2000 but negative on HEp2, showed reactivities: one with Ro52, one with SSB and one with Ro52, Ro60 and SSB (SSA-fluorescence pattern on HEp2000). A significant fraction of patients with positive LIA results who were negative on IIF had classical systemic disease. Detailed diagnostic and serological validation will be discussed. The SSA pattern was seen in 9% of the HEp2000 positive samples, all of them showed Ro52 or Ro60 positivity on LIA. When considering the total population, 79/495 were positive on LIA, of whom 73.4% were detected with HEp2 and 74.4% were detected with HEp2000.
Conclusion: IIF has no optimal sensitivity for detecting anti-ENA reactivities. In the context of clinical suspicion, anti-ENA testing is warranted, regardless of the IIF result.

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Induction of ANA, anti-dsDNA and anti-nucleosome antibodies following infliximab treatment in rheumatoid arthritis and spondyloarthropathy patients

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Background: Rheumatoid arthritis (RA) and spondyloarthropathy (SpA) patients are successfully treated with infliximab. There is indication that this therapy induces ANA and anti-dsDNA antibodies in RA.
Objectives: To investigate the induction of ANA, anti-dsDNA and anti-nucleosome antibodies in both RA and SpA after infliximab treatment.
Patients and methods: Sera from 62 patients with refractory RA treated with infliximab (3 mg/kg infliximab IV at week 0, 2, 6 and every 8 weeks thereafter) were obtained at baseline and week 30. Sera from 35 patients with active SpA treated with infliximab (5 mg/kg infliximab IV at week 0, 2 and 6 and 10 mg/kg every 14 weeks thereafter) were obtained at baseline and week 34. Samples were tested for ANA (indirect immunofluorecence [IIF] on HEp-2 cells), anti-dsDNA antibodies (IIF on Crithidia luciliae and Varelisa, PharmaciaDiagnostics), anti-nucleosome antibodies (Medizym anti-Nucleo, Medipan Diagnostica).
Results: At baseline, 32/62 RA and 6/35 SpA patients were positive for ANA. After infliximab treatment, an increase in ANA intensity of 2 or more steps (on a 0 to 5+ scale) was observed in 28/62 RA and in 26/35 SpA patients. A patient was defined as having developed anti-dsDNA antibodies when a positive result in both the IIF on Crithidia luciliae and anti-dsDNA ELISA was observed. At baseline, none of the RA and SpA patients had anti-dsDNA antibodies. After treatment, 7/62 RA and 6/35 SpA patients developed anti-dsDNA antibodies. At baseline, anti-nucleosome antibodies were observed in 1/62 RA and 3/35 SpA patients. After treatment, 5/62 RA and 4/35 SpA patients were positive for anti-nucleosome antibodies.

Conclusion: Infliximab treatment induced ANA and anti-dsDNA antibodies in both RA and SpA patients. After treatment, anti-dsDNA antibodies were significantly associated with anti-nucleosome antibodies.

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Membrane expression of neutrophil proteinase 3 (PR3) is associated with relapse in PR3-ANCA related vasculitis

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Background: The highly specific presence in serum of autoantibodies directed against intracellular neutrophil proteins such as PR3 (PR3-ANCA) suggests a pathophysiological role of these autoantibodies in patients with necrotizing small vessel vasculitis. A stable but interindividually highly variable membrane expression of PR3 has been found on resting neutrophils. We hypothesized that, in patients with PR3-ANCA related vasculitis, a higher expression of PR3 on neutrophil membrane would lead to more interaction with PR3-ANCA and could thereby influence the extent or course of the disease.
Methods: PR3 expression on unstimulated isolated neutrophils from patients with PR3-ANCA related vasculitis was determined by FACS analysis using anti-PR3 murine mAb 12.8. Patients were divided according to the distribution of neutrophil membrane PR3 in 3 groups: low, bimodal, and high. Disease extent at diagnosis was scored with the Birmingham Vasculitis Activity Score (BVAS). Actuarial relapse-free survival was calculated from diagnosis to the first relapse and compared between groups with the logrank test.
Results: 89 patients (age 49±16.6; 47 male/42 female) with PR3-ANCA related vasculitis followed at our department were included. At diagnosis, renal involvement was present in 52 (58%) and pulmonary involvement in 49 (55%) patients, BVAS was 23±10.5. During follow-up (81±67 months) 50 patients had one or more relapse. Age at diagnosis, organ involvement and BVAS at diagnosis were not different between patients with low (n=32), bimodal (n=26), and high (n=31) neutrophil membrane PR3 expression. However, median relapse-free survival was 104.5 months in patients with low PR3 expression as compared to 36.6 and 30.8 months in patients with bimodal and high PR3 expression, respectively ($P = 0.023$). Clinical manifestations at first relapse of vasculitis were not different between these groups.
Conclusion: The level of individual PR3 expression on resting neutrophils is significantly associated with risk for relapse in patients with PR3-ANCA associated vasculitis, but not with disease extent or manifestations at diagnosis or relapse. These data support the hypothesis that interaction *in vivo* of ANCA with PR3 expressed on neutrophil membrane plays a role in the pathophysiology of PR3-ANCA related vasculitis.

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Cryoglobulins induce temperature-dependent TNF-α production via Fcγ-receptor II

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Introduction: Cryoglobulins (CG) are antibodies that reversibly agglutinate and form immune complexes (IC) when plasma or serum is cooled below normal body temperature. Precipitation of CG is also known to depend on pH and ionic strength. We have previously shown that IC from SLE-patients can induce cytokine production from peripheral blood mononuclear cells (PBMC). We have investigated if, and under which circumstances, CG can also induce cytokine production.
Methods: CG from two patients were purified under sterile conditions. One patient was a 71 years old male with an IgG multiple myeloma and an IgG CG. The other patient was a 58 years old female with Waldentröm's macroglobulinemia and an IgM CG. The purified CG were added to PBMC cultures. Cytokine production in the supernatants was measured with ELISA. Fcγ-receptors were blocked with specific antibodies against FcγRII and FcγRIII. In separate experiments temperature, ionic strength and pH were varied.
Results: CG, especially IgG CG, induce production of TNF-α and IL-1β. This cytokine production was partly blocked by anti-FcγRII antibodies but not by antibodies to FcγRIII. When CG were added to PBMC at 4°C and 37°C respectively, more TNF-α production was seen in cultures with CG added at 4°C. The IgG and IgM CG showed maximal precipitation at different ionic strength, with parallel differences in TNF-α production in cell cultures with varying ionic strength. Parallel studies on the effects of pH on CG precipitation and cytokine production are underway.
Conclusions: CG-induced cytokine production varies with temperature, ionic strength and pH, and is at least partly mediated via FcγRII. CG-induced production of proinflammatory cytokines might be of importance in CG-associated vasculitis/nephritis. Modulation of CG precipitation and ensuing cytokine production may gain therapeutic importance.

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Assessment of activity and damage in ANCA-associated vasculitis in India

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Background: Wegener's Granulomatosis, once thought to be uncommon in India, is being recognized with increasing frequency in Indians. In the present study, the assessment of the primary systemic necrotizing Vasculitis was done using the Birmingham Vasculitis Activity Score (BVAS) and Vasculitis Damage Index (VDI).
Method: Seventy-six patients with ANCA-associated vasculitis were evaluated using the BVAS and VDI, between January 1990 and June 2001. The diagnosis of Wegener's granulomatosis and Churg Strauss disease were made by 1990 ACR criteria and that of Microscopic Polyangiitis by Chapel Hill Consensus. ANCA, ANA and anti-DNA were estimated by indirect immunofluorescence and antibodies to PR3 and MPO by ELISA. All other causes for secondary vasculitis and infections were excluded.
Results: There were 40 males and 36 females. The mean age at diagnosis was 43.4. The mean disease duration prior to diagnosis was 3.4 months. The distribution of vasculitis were: Wegener's granulomatosis, 48, microscopic polyangiitis, 10, Churg Strauss, 6 and crescentic glomerulonephritis, 12. cANCA was positive in 48(63.15%) and pANCA in 21(27.63%). ANA and anti-DNA were negative in all the patients. The mean BVAS score at baseline was 16.4. The mean VDI system score was 3 and the mean total VDI score was 4.6. Using the VDI, the following items of damage were seen: musculoskeletal damage, 12 (15.8%); skin damage, 16 (21%); ENT damage, 28 (36.8%); pulmonary damage, 42 (55.3%); cardiovascular damage, 34 (44.7%); renal damage, 51 (67.1%); peripheral vascular damage, 26 (34.2%); ocular damage, 21 (27.6%); neuropsychiatric damage, 48 (63.2%); and other damage & drug toxicity, 14 (18.4%).
Conclusions: 1) ANCA-associated vasculitis were rare in India, present in only 0.001% of Hospital admissions. 2) Neuropsychiatric manifestations were common (63%). 3) The BVAS and VDI offer a comprehensive and cumulative measure of disease activity and damage in the serial assessment of vasculitis patients.

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Clinical optimization and multicenter validation of antigen-specific cut-off values on the INNO-LIAr ANA Update for the detection of autoantibodies in connective tissue disorders

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Objectives: Optimization and validation of antigen-specific cut-off values for SmB, SmD, RNP-70k, RNP-A, RNP-C, SSA/Ro52, SSA/Ro60, SSB/La, Cenp-B, Topo-I, Jo-1, ribosomal P and histones to achieve 98% specificity for each of the markers.
Patients and methods: The INNO-LIAr ANA Update is a qualitative test detecting antibodies to several different antigens, most of which are recombinantly made, with the exception of SSA/Ro60 and histones (natural), and SmD and ribosomal P (synthetic). The LIA-SCAN ANA provides a quantitative read-out of the INNO-LIAr ANA Update results. The cut-off value of the different antigen lines was optimized using an in-house set of 955 samples. The assay specificity was validated at multiple sites using a different set of 330 samples obtained from 158 apparently healthy blood donors, 100 patients with a variety of infections, 20 with Wegener's granulomatosis, 20 with inflammatory bowel disease, 20 with primary antiphospholipid syndrome, and 12 with psoriatic arthritis. The INNO-LIAr ANA Update reactivity using the optimized cut-off was tested in 147 patients with scleroderma, 93 with Sjögren's disease, 40 patients with systemic lupus erythematosus (SLE), 40 with rheumatoid arthritis (RA), 39 with mixed connective tissue disease, and 19 with myositis. The clinical diagnosis was considered as the gold standard.
Results and Conclusions: The optimized cut-off values resulted in an average specificity of over 98% for all LIA markers in the validation set of 330 samples. The pattern of reactivity for the different LIA ANAs in the 378 samples from the target patient groups corresponded to the sensitivities reported in the literature. In conclusion, the INNO-LIAr ANA Update shows uniformly high specificities combined with sensitivities very similar to those of reference assays, in a single test format.

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Autoantibodies to deiminated fibrinogen are the most efficient serological criterion for the diagnosis of rheumatoid arthritis

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Background: Antifilaggrin autoantibodies are recognized as the most specific serum marker in rheumatoid arthritis (RA). Their high diagnostic value led in the past few years to the development of several efficient tests for detection of these autoantibodies directed against deiminated ('citrullinated') peptidic epitopes borne by profilaggrin. We recently showed that deiminated forms of the α and β chains of fibrin are the major targets of antifilaggrin autoantibodies in the synovial tissue of RA patients. Consequently, we developed a new test to assay the serum autoantibodies to deiminated fibrinogen (AhFibA) and evaluated its diagnostic value in RA.
Methods: An enzyme-linked immunosorbent assay (ELISA) using *in vitro* deiminated human fibrinogen as immunosorbent was developed, and 617 sera from patients with well characterized rheumatic diseases, including 181 RA, were tested. The diagnostic performance of the AhFibA-ELISA was compared, in the same cohort of patients, to that of rheumatoid factor (RF) assayed by nephelometry, and to those of various methods for detection of antifilaggrin autoantibodies: indirect immunofluorescence ('antikeratin antibodies'), immunoblotting onto human epidermis filaggrin, CCP-ELISA and an ELISA on deiminated recombinant rat filaggrin (ArFA-ELISA).
Results: With a cut-off value allowing 95% of specificity to be reached, AhFibA were detected in 83% of the RA sera. At 98.5% specificity, 76% of the RA sera were positive. In contrast, at the same specificities, RF was detected in only 56% and 6% of the RA sera, respectively. In addition, the diagnostic performance of the AhFibA-ELISA was significantly higher than those of all the tests for antifilaggrin autoantibodies, including CCP-ELISA and ArFA-ELISA.
Conclusions: Presence of autoantibodies to deiminated human fibrinogen appears as the most efficient (specific and sensitive) serological criterion for the diagnosis of RA. Moreover, these results confirm that deiminated fibrin is a major B autoantigen in RA and sustain its probable involvement in the pathophysiology of the disease.

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Characterization of binding interactions in solution between β-2-glycoprotein I and monoclonal antibodies

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The circulating so-called antiphospholipid antibodies associated with autoimmune thrombophilia appear in most cases to be directed against phospholipid-binding proteins, chiefly apolipoprotein H (β-2-glycoprotein I, β2gpI) or prothrombin. Accordingly, the determination of antibodies reacting with β2gpI in solid-phase immunoassays is useful in the diagnostic workup of thrombophilic patients. These antibodies are characterized by significant binding only when β2gpI is immobilized on "high binding", chemically activated, or phospholipid-coated surfaces. It is not yet known, however, if the binding of antibodies is a result of a specific conformation that β2gpI attains when immobilized on a surface or simply a reflection of a sufficiently high epitope density. We are establishing solution binding assays based on capillary electrophoresis to measure antibody–β2gpI interactions without immobilizing any of the interacting molecules. Here we describe the optimization of analysis conditions in uncoated and coated capillaries for native and succinylated human β2gpI. Monoclonal anti-β2gpI and the humanized monoclonal antibodies proposed as standards for solid-phase assays were used as model ligand systems and we demonstrate the measurement and quantitation of binding. The influence of changes in buffer conditions such as ionic strength, pH, and of buffer additives including heparin - a known β2gpI ligand - is easily assessed by this approach and minute amounts of biological material are consumed (nanoliter injection volumes). The set-up is well suited for determining weak affinity interactions and will be helpful in providing answers to the questions about solution affinities of patient antibodies for native β2gpI and thus for understanding the molecular pathology behind the thrombophilic effect of anti-β2gpI autoantibodies.

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Anti-nuclear antibody (ANA) analysis should include specific analysis of antibodies to SS-A/Ro

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Objectives: Evaluation of ELISAs and immunodiffusion (ID) in addition to IF microscopy for ANA screening.
Methods: 3079 patient sera sent for routine analysis were diluted 1:100 in PBS and examined by IF microscopy (HEp-2 cells) for the presence of ANA. In addition, all sera were analysed by two different commercial ELISAs for screening of ANA specificity. The sera were also analysed by an in-house ID test for precipitating antibodies to extractable nuclear antigens (ENA). All sera positive in any of the ELISAs or ID were further analysed by an 'ANA profile' ELISA kit and a commercial ID test for determination of antigenic specificity.
Results: 334 (11%) of the sera were positive by IF microscopy. 102 (31%) of these sera were also positive in at least one additional test. 214 HEp-2 negative sera (7%) turned out positive in at least one of the additional ANA tests. In 40 of these 214 sera the antigenic specificity was confirmed by the commercial ID test, 37 (92%) of which proved to have specificity for SS-A/Ro. The different ELISAs correlated poorly with one another.
Conclusion: In addition to IF-microscopy on HEp-2 cells, an assay for anti-SS-A antibodies should be included for routine ANA screening.

Cellular immunity

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Abnormal gene expression in CD8 T cells from the lungs of scleroderma patients

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Activated $CD8^+$ T cells in bronchoalveolar lavage (BAL) samples are associated with progressive lung fibrosis in scleroderma. The hypothesis of this work is that $CD8^+$ T cells from a subset of scleroderma patients, especially those with lung inflammation, have an abnormal pattern of gene expression that can promote fibrosis. Freshly isolated BAL $CD8^+$ T cells from 26 individuals were tested for expression of cytokines, chemokines, growth factors, and receptor genes, using DNA microarrays. Hierarchical cluster analyses showed two groups of arrays. Group 1 included all 10 patients with lung inflammation and two patients without. Arrays from the other 7 patients without lung inflammation clustered with all 7 arrays from controls in group 2. Differences in gene expression indicated that $CD8^+$ T cells in group 1, compared to group 2, were more likely to be activated, express type 2 cytokines, and stimulate extracellular matrix deposition, and less likely to undergo activation-induced cell death. T-cell activation was suggested by increased expression of LIGHT, neurotropin-4, CD100, CD6, integrin b1 and decreased expression of VIP R1 genes. Type 2 cytokine production was suggested by increased expression of CCR4, G-CSF R and IL-13 Ra and decreased expression of TRANCE and TNF R1 genes. Reduced expression of TNF R1, TNF R11, TRAIL R1, CD30 L and Fas genes suggested that CD8+ T cells from Group 1 were less likely to die following activation. Increased gene expression of oncostatin M, which simulates fibroblast proliferation and collagen production, integrin b6, which can activate TGF-β, FGF17, FGF4, and membrane type-1 and -2 matrix metalloproteinases was seen. To confirm some of these results, increased IL-4 mRNA had been reported in our previous work, and increases in oncostatin M and activated TGF-β proteins in BAL fluids from patients with lung inflammation were confirmed by ELISA. These findings suggest that activated $CD8^+$ T cells are part of a pathway that leads to lung fibrosis in scleroderma.

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CD25 regulatory cells are involved in protection from collagen-induced arthritis in both susceptible and non-susceptible mice

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$CD4^+$ regulatory T cells expressing the activation marker CD25 have been strongly implicated in the control of autoimmune diseases. For example, immune compromised mice that receive splenocytes depleted of $CD25^+$ cells develop a variety of organ-specific autoimmune diseases such as colitis, gastritis, oophoritis, orchitis, and thyroiditis. Nonetheless, the role of regulatory T cells in many other autoimmune diseases, particularly systemic autoimmune diseases, has not been explored. We have now analyzed the role of $CD25^+CD4^+$ T cells in the control of collagen-induced arthritis (CIA), a commonly accepted model of rheumatoid arthritis. Depletion of $CD25^+$ T cells in CIA susceptible DBA/1 mice leads to an earlier and more aggressive disease as evidenced by the development of severely swollen limbs following collagen injection. These mice also showed an increase in collagen specific antibodies. In addition, B6 mice, which are normally not susceptible to CIA, rapidly develop arthritis when vaccination is performed after depletion of $CD25^+$ cells. Together, these findings indicate the importance of $CD25^+$ cells in modulating CIA, establishing them as significant contributors in the control of chronic and systemic joint-related inflammation like that found in rheumatoid arthritis.

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The accumulative and maturative type of B-cell activation in synovitis: a new concept of B-cell activation in autoimmune diseases

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Introduction: B-cells and plasma cells in chronic synovitis of osteoarthritis (OA), reactive arthritis (ReA) and rheumatoid arthritis (RA) show two different histological patterns: lymphoplasmacellular pattern and germinal centre (GC) pattern. To clarify what immunological mechanisms stands beyond these histological patterns, IgVh gene analysis from the B and plasma cells present in those patterns was performed.
Methods: IgVh gene repertoires were determined from RNA (OA=5, ReA=2, RA=5), prepared from tissue cryosections and from isolated B-cells and plasma cells (plc) by micromanipulation. The B-cell environment was analysed immunohistochemically by detecting plc (IgM, IgG, IgA), B-lymphocytes (CD20) and Ki-67 positive (proliferating) cells.

Results: In the lymphoplasmacellular pattern of OA (n=5), somatically highly mutated IgVh genes (R/S: CDR=R/S=5.3; Fr=R/S=2.0) could be detected, but no clonal relation could be established amongst them. The plc of the GC pattern in ReA (n=2) and RA (n=5) revealed highly somatically mutated IgVh genes: R/S: CDr=R/S=3.4; Fr=R/S=1.7; and $CD20^+$ B cells in germline configuration. A clonal relation between low mutated B cells and highly mutated plc could be established indicating a local clonal expansion. A proliferation of $CD20^+$ B cells could only be observed in GC.
Conclusions: Therefore, two different patterns of B-cell activation may be defined: (1) The accumulative type: already activated B cells migrate into the synovial tissue and accumulate there without further IgVh diversification. (2) The maturative type of B-cell proliferation with IgVh diversification occurring in a GC like reaction. The two different patterns of B-cell activation may reflect that different antigens are involved in autoimmune and degenerative diseases. Since in organ specific autoimmune diseases (RA, Morbus Sjögren, Morbus Basedow) a local germinal centre reaction takes place, it is likely that the B cells are expanded by disease specific antigens. The experimental expression of IgVh genes isolated from these germinal centre B cells may therefore lead to the identification of pathogenic organ specific antigens.

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Regulation of T cell and monocyte activation by human anergic/suppressive CD4 CD25 T cells

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Regulation of the immune response is important to avoid chronic inflammation and autoimmunity. Anergic/suppressive $CD4^+CD25^+$ T cells have been shown to be major contributors to this regulation. We have previously shown that the suppressive $CD4^+CD25^+$ T cells can suppress mitogenic and antigen-specific $CD4^+$ T-cell responses in humans (*EJI* 2001, **31**:1122; *Immunology* 2001, **104**:6). In rheumatoid arthritis (RA), besides $CD4^+$ T cells also monocytes play an important role in the disease process. Therefore we investigated whether the suppressive $CD4^+CD25^+$ T cells could affect monocyte activation as well as T-cell activation. $CD4^+$ T cells and monocytes were isolated from peripheral blood mononuclear cells from healthy donors via MACS isolation techniques. $CD4^+$ T cells were separated into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. T cells and monocytes were cocultured for two days without or with anti-CD3 mAb, after which proliferation, cytokine production and phenotypic markers were investigated. Coculture of monocytes with $CD4^+CD25^-$ T cells in the presence of anti-CD3 mAb resulted in strong T-cell proliferation, production of cytokines (TNF-α, IFN-γ, IL-1β, IL-10), upregulated expression of HLA II, CD40 and CD80 and remaining high CD86 levels on the monocyte/macrophage. In contrast, coculture of monocytes with $CD4^+CD25^+$ T cells and anti-CD3 mAb resulted in T-cell anergy, low levels of cytokine production, reduced upregulation of HLA II, CD40 and CD80 and downregulation of CD86. We are currently investigating how this altered activation affects the antigen-presenting and/or cartilage-destructive capacity of the monocytes/macrophages. In conclusion, we demonstrate that anergic/-suppressive $CD4^+CD25^+$ T cells can suppress – besides T-cell responses – also the activation of monocytes. Further investigation into the function of these suppressive $CD4^+CD25^+$ T cells in RA patients as well as understanding their regulatory mechanism might elucidate their potential role in the pathogenesis of RA.

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The characterisation and regulation of type 1 immune responses in psoriatic arthritis

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Psoriatic arthritis (PsA) is a disabling inflammatory disease, of as yet unknown aetiology. A pathogenic role for TNF-α is suggested by the beneficial effects of anti-TNF therapy. However, upstream events remain to be elucidated. We propose that elevated levels of IFN-γ expression in inflamed PsA tissues, reflects a 'type 1' immune response, of pathophysiological significance. This study sought to identify and phenotype spontaneous IFN-γ secreting cells within PsA inflammatory lesions. Furthermore, we have elucidated factors, including costimulatory molecules and innate cytokines that regulate IFN-γ expression.
Methods: Matched PsA and rheumatoid peripheral blood (PB) and synovial fluid (SF) mononuclear cells were isolated on density gradients. Spontaneous IFN-γ secreting cells were identified using a novel bi-specific antibody capture method. T cells were further phenotypically characterised by FACS analysis. Following *in vitro* stimulation with mitogen/cytokines mononuclear cells were analysed for cytokine production by ELISA. Immunohistology on both skin and synovial membrane samples was performed using standard methods.
Results: We have identified an increase in the percentage of spontaneous IFN-γ secreting cells in PsA synovial fluid when compared to rheumatoids. FACS analysis of PsA SF further identified cells expressing both CCR5 and IL-18R, characteristic of a Th1 phenotype. The presence of IL-12 and IL-18, potential inflammatory mediators, was confirmed in synovial membrane samples by immunohistochemistry. Furthermore, *in vitro* cultures of matched PsA PB/SF cells showed enhanced IFN-γ production by SF cells following stimulation with recombinant IL-12/IL-15. A threefold increase of IFN-γ was detected in the presence of CD3/CD28/IL-12 in SF compared with PBMC and a twelvefold increase in the presence of CD3/CD28/IL-15.
Conclusions: We have detected the presence and defined functional significance for the candidate regulatory cytokines, IL-12, IL-15 and IL-18 in sustaining IFN-γ expression. These findings support the hypothesis that PsA pathogenesis is associated with a 'type 1' polarised immune reponse.

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Identification of T-cell specific autoantigens

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The mechanisms that lead to the formation of autoantibodies are not understood. Accumulating data suggest that autoantigens might be characterized by post-translational modifications that are part of the natural apoptosis process such as granzyme B cleavage (i.e. topoisomerase I, LA (SS-B), U1-70 kd), caspase cleavage (i.e. topoisomerase I and II), phosphorylation/dephosphorylation (i.e. ribosomal P proteins, LA (SS-B), transglutaminase crosslinking (i.e. histone H2B) and deimination (arginine–citrullin change in fibrin or fibrinogen). T cells, which have been negatively selected or tolerized against native autoantigens, might be primed by modified autoantigens and deliver help to self-reactive B cells. So far, there have been only few reports about autoantigen specific T cells. Since the frequencies of those T cells in the peripheral blood are very low, autoantigen specific T-cell responses are difficult to

detect. In our study we are combining the use of monocyte derived dendritic cells (DC) as professional antigen presenting cells with intracellular cytokine staining for the detection of T-cell responses. Monocytes and lymphocytes are purified from freshly isolated PBMC by density gradient centrifugation. Lymphocytes are frozen in liquid nitrogen until usage. Monocytes are differentiated into immature dendritic cells in culture medium containing either 1% FCS or 10% autologous serum and GM-CSF and IL-4. Maturation is induced by addition of GM-CSF, LPS, TNF-α and IL-1-β in the presence or absence of a specific antigen. Mature DC expressing antigen derived peptides are washed and cocultured with thawed autologous lymphocytes. After 4 hours, brefeldin A is added and lymphocytes are intracellulary stained with CD3, CD4, CD69 and TNF-α mAb after o/n culture. We will use this system to identify possible T-cell specific autoantigens by using granzyme B cleaved nuclear extracts and purified modified nuclear antigens.

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Tolerance induction in mice by antibodies to T-cell epitopes

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CD4/DR3 mice lack murine cd4 but express human CD4 specifically on helper T cells, and HLA-DR3 as its natural counter ligand (CD4/DR3 mice). The injection of these mice with anti-human CD4 prior to immunization with tetanus toxoid (TT, day 0) totally blocked the formation of specific Abs. When these mice were left untreated for at least 30 days, and were then re-exposed with TT, but in the absence of anti-human CD4, they consistently failed to induce specific antibodies (long-term unresponsiveness). Importantly, the concurrent injection of TT and anti-human CD4 at day 0, followed by another two anti-CD4 treatments also led to tolerant animals indicating that tolerance was inducible as late as the antigen exposure is provided. We demonstrate a limited ability of spleen cells to respond to TT *in vitro* indicating that T cells are essentially involved in the maintenance of tolerance. These data show for that the human CD4 coreceptor may mediate tolerance-inducing signals when triggered by an appropriate ligand *in vivo*.

Innovative therapies

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Non viral gene therapy in arthritis by *in vivo* intramuscular IL-10 DNA electrotranfer

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Intramuscular electroporation of DNA is an attractive technique for nonviral gene transfer of therapeutic genes in inflammatory/autoimmune disease such as rheumatoid arthritis (RA). We have developed *in vivo* electroporation for efficient cytokine gene transfer in collagen induced arthritis.
Methods: We co-injected in the tibialis anterior of DBA1 mice a standard 30 ml dose of plasmid DNA encoding the anti-inflammatory cytokine viral interleukin-10 (vIL-10) under the control of a doxycycline-inducible promoter, and a plasmid expressing the tetracycline controlled transcriptional silencer (tTS) that binds promoter in absence of doxycycline. Electroporation was performed *in vivo* using 8 pulses of 200 v during 1 ms day 25 postimmunization of DBA1 mice with collagen type II.
Results: Electroporation resulted in a dose-dependent increase in the vIL-10 expression in muscle and serum. The transgene was expressed only by muscle cells during 4 weeks. The doxycycline treatment showed significant inhibitory effects on DBA1 mice type II collagen induced arthritis (CIA) as paw swelling was reduced (1.79±0.22 vs. 2.13±0.84 mm on day 32 postimmunization) and onset of arthritis clinical delayed in the doxycycline-treated group compared with the control group without doxycycline (32.62±4.50 days, versus 28.38±3.62 days respectively).
Conclusions: Muscle-targeted vIL10-rtTA plasmid transfer by *in vivo* electroporation is a suitable approach for non viral gene therapy in arthritis.

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Alefacept treatment in psoriatic arthritis: reduction of the synovial inflammatory infiltrate and improvement of clinical signs of arthritis

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Objective: Psoriasis and psoriatic arthritis (PsA) are thought of as T-cell mediated diseases. LFA-3/CD2 interaction plays a significant role in T-cell activation. Alefacept, an LFA3-IgG1 fusion protein, blocks LFA3-CD2 interactions resulting in inhibition of T-cell responses and T-cell apoptosis which could be beneficial in patients with active PsA.
Methods: Eleven patients with active PsA were treated with alefacept for 12 weeks in an open label design. Clinical joint assessment, Psoriasis Area and Severity Index (PASI), and peripheral blood (PB) assessments were performed at baseline, after 4, 9, 12, and 16 weeks of treatment. Serial synovial tissue (ST) biopsies of an index joint (knee, ankle, wrist or MCP joint) were obtained by arthroscopy at baseline, 4 and 12 weeks.
Results: At completion of treatment 6 out of 11 (56%) treated patients fulfilled the DAS response criteria, 9 patients (82%) fulfilled the DAS response criteria at any point within the study. Seven of 11 (64%) treated patients showed improvement (mean 50%) of their skin psoriasis. In the ST there was a statistically significant reduction in CD4+ lymphocytes ($P < 0.05$), CD8+ lymphocytes ($P = 0.05$), and CD68+ macrophages ($P < 0.02$) in the synovial samples after 12 weeks of treatment compared to baseline. Patients fulfilling the DAS response criteria demonstrated a higher baseline ration and significant reduction in CD4+CD45RO+ cells in both ST and PB where non-responders demonstrated only reductions in PB.
Conclusion: The improvement in clinical joint score, skin psoriasis, and changes in synovial tissue after treatment with alefacept supports the hypothesis that T-cell activation plays an important role in this chronic inflammatory disease. Furthermore, since alefacept, a specific T-cell agent, led to decreased macrophage activation, the data indicate that T cells orchestrate synovial inflammation in psoriatic arthritis.

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Chemokine blockade in patients with rheumatoid arthritis: reduction of synovial inflammation

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Objective: Leukocyte migration to the synovial compartment plays an important role in rheumatoid arthritis (RA). The presence of macrophages in the synovium is associated with disease activity (*Arthritis Rheum* 1997; **40**:217-225). Important mediators of leukocyte migration include chemokines and their receptors. This study evaluated the effects of blocking the migration of monocytes/macrophages and T cells into the joint using a chemokine receptor antagonist.
Methods: 16 patients were included in a 2-week, double-blind, placebo-controlled randomised phase-Ib-study evaluating the effects of an oral CCR1 antagonist. Synovial tissue was obtained by arthroscopy on day 1 and day 15. Immunohistochemistry was performed to detect CD68, CD3, CD4, CD8, CD55, CD22, and CD138. Sections were analysed using digital image analysis. The results before and after treatment were compared by paired, non-parametric analysis (Wilcoxon signed rank test). A two-sample *t*-test was used to compare the changes from baseline in the two groups (12 patients in the treatment group versus 4 patients in the placebo group).
Results: After CCR1 blockade, there was a statistically significant decrease in: overall cellularity (reduction of 2065±256 (median±s.e.m.) to 891±164, $P < 0.02$), intimal lining layer hyperplasia (269±95 to 181±49, $P < 0.02$), CD68+ macrophages (2386±261 to 1445±283, $P < 0.02$), intimal CD68+ macrophages (1201±130 to 496±133, $P < 0.03$) and CD4+ T cells (432±160 to 215±139, $P < 0.02$). There also was a decrease in CD8+ T cells (131±44 to 35±31), but the difference was not statistically significant. As expected there was no decrease in the number of CD22+ B cells, CD138 plasma cells, or CD55+ fibroblast-like-synoviocytes, since these cells do not express CCR1. There was on average no change in the features of synovial biopsy samples from patients who received placebo.
Conclusion: It is feasible to influence the migration of inflammatory cells to the joints by using an oral CCR1 antagonist. This could provide a completely new treatment for RA patients.

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High dose cyclophosphamide followed by autologous stem cell transplantation for the treatment of intractable rheumatoid arthritis (RA): a 2-year clinical and immunological follow-up

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Background: To investigate the effects of high dose chemotherapy (HDC) and autologous stem cell transplantation (ASCT) both clinically and on the synovial infiltrate for selected patients with severe, refractory RA.
Methods: 12 patients with rheumatoid arthritis, were treated. Mobilization of autologous blood stem cells was accomplished with cyclophosphamide (4 g/m^2) and G-CSF. The conditioning regimen consisted of intravenous administration of high dose cyclophosphamide (totalling 200 mg/kg), with subsequent reinfusion of the positively selected graft. Biopsies of synovial tissue from a knee were obtained before and three months after HDC and ASCT. Immunological monitoring and immunohistochemistry on the synovial infiltrate was performed.
Results: The procedure appeared feasible in all patients. The aplastic period lasted less than 4 weeks in all patients. Efficacy data, with follow-up ranging from 6–24 months showed that the mean disease activity score (DAS) decreased from 5.4 (n=12) to 3.2 at 6 months (n=12, $P = 0.003$), 3.1 at 12 months (n=11, $P = 0.005$) and 2.9 at 24 months (n=5, $P = 0.043$). Mean DMARD-free period was 12.9 months (95% CI: 7.23–18.48). Patients could be classified in clinical responders (Good response (Eular); ACR >50%, n=6) and non-responders (Moderate or no response; ACR ≤20%, n=6) at three months post-transplantation. Immunophenotyping of PBMCs showed prolonged (>24 months) depletion of CD45RA+ T cells after transplantation, whereas levels of CD8+, CD3-CD16CD56+ and CD19+ cells quickly recovered. CD4+CD45RO+ cells were not complety depleted after transplantation. High IgG1 in peripheral blood (responders: 9.69 g/l; non-responders 6.24 g/l $P = 0.046$) and high baseline synovial CD27 (mean infitration score; 3 vs. 0.33; $P = 0.036$) and CD45RO (mean infiltration score; 3.4 vs. 0.67; $P = 0.036$) predicted clinical response. Furthermore there were trends towards a decrease in T-cell markers in the synovium before and after transplantation were compared (CD3-CD4-CD25-CD45RA-RO and CD27), but no statistical significant differences were found. IL-1 showed significant higher scores in non-responders than responders after transplantation: 3.5 vs. 0.6 ($P = 0.024$).
Conclusions: The results of the present interim-analysis underscore the feasibility and potential efficacy of HDC followed by ASCT for the treatment of intractable RA. Clinical effect of HDC and ASCT correlated with T-cell debulking in synovial tissue.

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Mycophenolate mofetil prevents the development of a clinical relapse in SLE patients at risk: an open pilot study

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Background: Systemic lupus erythematosus (SLE) is characterised by the presence of antibodies to double-stranded DNA (dsDNA). These antibodies are supposedly involved in the pathogenesis of SLE. Eighty percent of patients develop a clinical relapse within 10 weeks after a significant rise in anti-dsDNA level. This can be prevented by the administration of corticosteroids at the time of rise in anti-dsDNA. We hypothesise that administration of mofetil mycophenolate (MMF) will have similar effects without the side effects of corticosteroids.
Methods: SLE patients (n=36) were followed monthly for a rise in levels of anti-dsDNA, defined as exceeding 125% of the level of the previous sample, and amounting at least 15 E/ml within a 4-month period. At the time of a rise patients started with 2000 mg MMF daily for a period of 6 months. Patients were monitored monthly for the occurrence of a clinical relapse and to assess serological activity and state of activation of CD4+, CD8+ and CD19+ lymphocyte subsets.
Results: In 10 patients a serological relapse was encountered. All patients started MMF and completed a 6 months study period without the occurrence of a clinical relapse. Side effects were minimal. Antibodies to dsDNA decreased during the study period ($P < 0.001$) associated with a decrease in activation of CD19+ lymphocytes. No difference in the state of activation of CD4+ or CD8+ lymphocyte subsets could be demonstrated.
Conclusion: Administration of MMF after a rise of antibodies to dsDNA prevents the occurrence of clinical relapses of SLE and is well tolerated.

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Reduction of cartilage destruction in a rapid progressive arthritis model in SCID mice

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Fibroblasts are considered to be a crucial cell population for disease progression, as well as joint destruction in rheumatoid arthritis. Recent data underline the potency of rheumatoid synovial fibroblast-like cells to induce the destruction of cartilage and bone, e.g. following intra-articular injection into SCID mice. We have isolated a fibroblastoid cell line by co-cultivation of human rheumatoid fibroblasts with murine fibroblasts. The generated cell line exhibits characteristics of rheumatoid fibroblasts and genetic alterations indicating a transformed phenotype. These cells have been shown to induce a rapid destruction of articular cartilage following intra-articular instillation. Fibroblastoid cells (LS48) were examined for cytogenetic characteristics, morphology, surface molecules, cytokine secretion, and functional parameters. 500,000 cells were injected directly into SCID mouse knee joints to induce cartilage destruction. Mice were monitored for joint swelling, serological parameters and by radiological methods. Furthermore, the effects of immunosuppressive drugs such as cyclosporine A, methotrexate, and FK 506 were investigated in this model. In addition, transfection of LS48 was performed with IL-11 as well as IL-15 prior to arthritis induction to investigate influence on cartilage destruction. Finally, the histology of cartilage destruction was explored. LS48 shows characteristics of a fibroblast-like cell but is of murine origin. Secretion of matrix metalloproteinases 3, 9 and 13 as well of interleukine-6 and tumor necrosis factor revealed similarities to human invasive rheumatoid synovial membrane fibroblasts. Rapid progressive cartilage destruction within 10 days was induced by instillation into SCID mouse knee joints. Morphology revealed invasion of fibroblast-like cells into the articular cartilage. Destruction could be reduced by methotrexate but not by cyclosporine A or FK 506, indicating a fibroblast-directed action of methotrexate connected to reduction of joint destruction in RA patients. Transfection with cytokines did not act on cartilage destruction, but IL-11 reduced apoptosis of chondrocytes. Induction of cartilage destruction by intra-articular application of murine fibroblast-like cells, in particular LS48, is a rapid and highly reproducible model for investigating invasive arthritis and can be modulated by drugs or gene transfer. This provides the opportunity to check novel therapeutic strategies for the treatment of arthritis, especially focussing on the reduction of cartilage erosion.

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Selective elimination of inflammatory macrophages by Fcγ-RI-directed immunotoxins: a novel concept in the treatment of rheumatoid arthritis

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Macrophages contribute to joint inflammation in RA by a number of functions. The type I IgG receptor (FcγRI) has been shown to be upregulated on inflammatory macrophages and can very efficiently endocytose FcγRI-targeted antigens. Based on its unique function and distribution it was tried to selectively eliminate inflammatory macrophages by a FcγRI-directed humanized antibody (H22) conjugated to the toxin Ricin A (anti-FcγRI-RiA).

FcγRI expression (CD64) was determined on RA monocytes/macrophages (mo/mac's) and granulocytes from peripheral blood (PB) and synovial fluid (SF). Anti-FcγRI-RiA was tested on mo/mac's from PB and SF of 12 RA patients. Cell death of mo/mac's (vs. lymphocytes) was assessed by morphological changes, changes in CD14 expression and nuclear DNA fragmentation (measuring apoptosis) after 24 hours. The anti-inflammatory effect *in vitro* was tested by the effect of anti-FcγRI-RiA on TNFα production, antigen-induced Th1-mediated proliferation and T-cell survival in the context of SFMC. In addition the effect on cytokine production by synovial tissue explants was studied (n=6).

FcγRI was exclusively expressed on mo/mac's and was higher in SF than PB (MFI 232 vs. 59, $P < 0.01$). Treatment with anti-FcγRI-RiA reduced high FcγRI-expressing mo/mac's and was associated and was associated by selective cell death of mo/mac's (death of lymphocytes was not observed after 24 hours). Immunotoxin-induced cell death was higher in SF than in PB (15% in PB vs. 61% in SF, $P < 0.01$). High expression of FcγRI correlated with increased capacity of anti-FcγRI to induce mo/mac cell death. Induction of macrophage cell death was mediated via apoptosis as measured by nuclear DNA fragmentation. After prolonged culture (3–5 days) macrophage death was associated with significant reductions in TNFα production, Th1-induced lymphocyte proliferation and T-cell survival in the context of SFMC (42%, 63%, 72%, respectively). Furthermore, IL-1 production by RA synovial tissue explants was inhibited (63%, $P < 0.01$). Macrophages from RA patients can be selectively eliminated by FcγRI-directed immunotoxins. Considering the important role of these cells in RA, this type of elimination may be a novel concept in the treatment of RA.

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Long term immune reconstitution after immunoablation and autologous CD34 cell therapy in autoimmune diseases

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Introduction: Immunoablation in combination with autologous stem cell transplantation (ASCT) is used as a therapy for severe autoimmune diseases. We have analysed reconstitution of the immune system in patients treated with ASCT.

Results: During a follow-up period of up to 42 months after ASCT, one polychondritis and two systemic lupus erythematosus (SLE) patients in clinical remission were analysed for reappearance of naive, activated and memory B and T lymphocytes, for reactivity against pathogens and autoantigens, and for presence of autoantibodies. Titers of disease-specific autoantibodies decreased after ASCT with the half-life of secreted antibodies, and did not reappear. Naive T and naive B cells reappeared and reached normal levels within one year after ASCT. T cells activated and expanded by pathogens were easily detectable, but not T cells reacting to any of an array of autoantigens tested, in a cytometric cytokine provocation test. A third SLE patient suffered from a relapse of disease after

being free of any clinical and serological symptoms for 17 months. In this patient, autoantibodies with old (anti-dsDNA antibodies) and new specificities (anti-Sm and anti-U1RNP antibodies) appeared upon relapse. A sudden decrease of peripheral naive and increase of peripheral memory B and Th cells preceeded the relapse.
Conclusion: ASCT for autoimmune diseases can result in longlasting remissions. According to frequencies and phenotypes of naive lymphocytes, the reconstituted immune system resembles a 'juvenile' immune system. Our results reveal that autoreactive and plasma cells do not survive the therapy but protective immune memory has to be re-established.

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A new non-viral vector for gene-therapy in rheumatic diseases

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Background and Objective: Treatment of rheumatoid arthritis (RA) is problematic with current strategies. Relatively high systemic doses are necessary to achieve therapeutic levels of antirheumatic drugs in the joints. Gene therapy might provide a more efficient system to deliver therapeutic compounds at the site of inflammation. Artificial Chromosome Expression System (ACes) is a unique non-integrating, non-viral gene expression system, which functions like a natural chromosome. This technology offers advantages over current expression systems because it allows stable and predictable expression of genes producing single or multiple proteins over long periods of time. We are developing *ex vivo* gene therapy using a murine artificial chromosome containing a reporter gene (LacZ) for local delivery of genes in rats with adjuvant arthritis. The aim of this study was to evaluate the transfection efficiency of ACes complexed to two commercially available transfection agents into primary cells, such as, rat skin fibroblasts (RSF).
Methods: Transfer efficiency and optimal dose of transfection agents was determined using iododeoxyuridine (IdUrd)-incorporated ACes complexed to LipofectAMINE PLUS (Life Technologies) and Superfect (Qiagen) (Cytometry Vol 44:100–105, 2001). Following transfection, the ACes were antibody labelled and the cells were analyzed by FACs for FITC-fluorescence and microscopic staining. Using optimised transfection conditions, hygromycin resistant colonies were expanded and stable, ACes containing, karyotypes were verified by FISH analysis. In addition, β-galactosidase expression was determined to monitor the expression of the reporter gene in the transfected cells.
Results: The delivery of intact artificial chromosomes was detected within 24 to 48 hours post transfection. Maximum delivery rates of 25% were observed. Flow cytometry data correlated well with microscopic observations. After growing the cells under hygromycin B selection, clones expressing LacZ were identified. Stability of the clones is currently under observation.
Conclusion: These data suggest that artificial chromosomes may have potential in *ex vivo* gene therapy applications using non-viral delivery techniques. Primary cells can be efficiently transfected with ACes and express the transgene. At present we are investigating local delivery of transfected cells to the joints of rats with adjuvant-induced arthritis.

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Effects of TIMP-1 and TIMP-3 gene transfer on invasiveness, proliferation and apoptosis of rheumatoid arthritis (RA) synovial fibroblasts (RA-SF)

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TIMPs play a key role in counter balancing the action of MMPs and have been associated with cell proliferation, inhibition of angiogenesis and induction of apoptosis. Here, we investigated the effects of TIMP-1 and TIMP-3 gene transfer on cartilage invasion, proliferation and apoptosis of RA-SF. RA-SF were transduced with an adenoviral vector expressing human TIMP-1 (AdTIMP-1) or TIMP-3 (AdTIMP-3). Transduction efficacy was assessed by LacZ staining of RA-SF that were transduced with an adenoviral β-galactosidase construct. Untransduced and mock transduced RA-SF were used as controls. TIMP-1 was measured by ELISA in the culture supernatants of AdTIMP-1 transduced and mock transduced cells every 10 days until 60 days after transduction. Proliferation was assessed by ^{3}H-thymidine incorporation, and the rate of spontaneous apoptosis as well as FasL induced cell death was determined by a histon fragmentation assay. AdTIMP-1 and AdTIMP-3 transduced RA-SF and control RA-SF were co-implanted with human articular cartilage under the renal capsule of SCID mice for 60 days and their invasiveness was evaluated on paraffin sections using a semiquantitative score. Transduction efficacy was 67%, and TIMP-1 levels in the supernatants of AdTIMP-1 transduced cells were 51.5±6.5 μg/ml as compared to 8.7±3.4 μg/ml in the mock transduced cells. These levels of TIMP expression were maintained for at least 60 days. AdTIMP-1 and AdTIMP-3 gene transfer resulted in an inhibition of proliferation (35% and 40% vs. mock, respectively; $P < 0.05$). Transduction of RA-SF with AdTIMP-3 but not TIMP-1 increased spontaneous apoptosis (+24%; vs. mock, $P < 0.05$) as well as susceptibility to FasL-induced cell death (+23% vs. mock, $P < 0.05$). In the SCID mouse model, untransduced and mock transduced RA.SF deeply invaded the cartilage (scores: 2.5±0.2 and 3.2 respectively). In the AdTIMP-1 and AdTIMP-3 transduced RA-SF, invasion was inhibited clearly (scores 0.9±0.4 and 1.2±0.2 respectively) Both AdTIMP-1 and AdTIMP-3 gene transfer inhibit proliferation of RA-SF and reduce cartilage invasion. In contrast to TIMP-1, adenoviral gene transfer with TIMP-3. has a strong pro-apoptotic effect on RA-SF and facilitates Fas mediated cell death. These results indicate that gene transfer of TIMPs may be a useful approach to inhibit joint destruction in RA.

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Suppression of *in vitro* and *in vivo* parameters of inflammatory synovitis by simvastatin

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We have explored *in vitro* and *in vivo* the immunomodulatory activities of simvastatin, an HMG Co-A reductase (HMGR) inhibitor, in the context of inflammatory arthritis.

Methods: Lymphocyte/monocyte populations were purified from peripheral blood (PB) and synovial fluid/tissues (SF/ST) from rheumatoid arthritis (RA) patients or normal controls as appropriate. Fibroblast-like synoviocytes (FLS) were obtained by serial culture from RA ST and utilised from passage 3. T cells were mitogen or cytokine (IL-15) activated then fixed in PFA prior to co-culture with macrophages. Collagen-induced arthritis (CIA) developed in male DBA/1 mice on d26 following priming (d0)/ip challenge (d21) with type II collagen in CFA.
Results: *In vitro*, simvastatin significantly suppressed macrophage TNFα release following cell contact with cytokine or mitogen activated T cells whether derived from normal or RA PB or from RA SF. Constitutive release of IL-6 by RA FLS was dose dependently suppressed by simvastatin ($P < 0.05$). *In vivo*, simvastatin administration (up to 40 mg/kg ip, n=12/group) from d21 reduced plasma cholesterol by 20% and prevented the development of CIA in a dose dependent manner in comparison with injection of drug vehicle alone ($P < 0.01$). *Ex vivo* analysis indicated significant suppression of collagen-specific humoral and cellular immune responses. Moreover, administration of simvastatin (40 mg/kg) significantly suppressed established arthritis (n=20/group) compared with drug vehicle ($P < 0.01$).
Conclusions: Simvastatin modulated the release of cell-contact induced proinflammatory cytokines *in vitro* from cells of RA PB and synovial origin. Both developing and importantly, established CIA were significantly suppressed by administration of simvastatin *in vivo*. These data demonstrate for the first time the anti-inflammatory, therapeutic potential of HMGR inhibitors in inflammatory arthritis.

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Electrotransfer of low doses of plasmid encoding interleukin-10 in gene therapy of collagen-induced arthritis

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Gene therapy is extremely promising in rheumatoid arthritis (RA). Electrotransfer (ET) is a recent method reported to enhance the *in vivo* effects of intra-muscular DNA injection. Interleukin-10 (IL-10) has anti-inflammatory effects in RA and in collagen-induced arthritis (CIA), a murine model of RA. We used ET to enhance penetration into skeletal muscle of plasmids encoding IL-10. CIA was induced in DBA/1 mice by immunization with bovine type II collagen. Injection into the tibial cranial muscle of low-dose (200 ng) pCOR plasmid encoding murine IL-10 (pCOR-CMV-mIL-10) was immediately followed by application of square-wave electric pulses (8 pulses of 200 V/cm, 20 ms duration at 2 Hz). Control groups received empty plasmid or saline before ET. When ET was performed twice on days 10 and 25 post-immunization, CIA was significantly delayed ($P < 0.05$) and attenuated ($P < 0.001$) in pCOR-CMV-mIL-10 ET groups vs. control groups. When pCOR-CMV-mIL-10 ET was started at disease onset (days 25 and 40), the clinical severity of CIA was reduced ($P < 0.05$). All groups treated early or late by pCOR-CMV-mIL-10 ET showed dramatic suppression of histological signs of arthritis compared to control groups. Taken together, these data indicate that administration of an anti-inflammatory gene by ET of naked DNA is effective *in vivo* in an arthritis model in preventive and curative protocols, even when low doses were given.

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A simple system for rapid generation of recombinant adenoviruses by ligation

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Recombinant adenoviruses provide a versatile tool for gene expression, and both ease of production and ability to infect non dividing cells have led to their widespread use in arthritis models. Classically, their construction is time-consuming, requires homologous recombination in permissive cells, and, despite recent technical improvements such as homologous recombination in bacteria, their construction remains often difficult with manipulation of cosmids or large plasmids containing full-length adenoviral genome. We have designed and constructed a system where recombinant adenoviruses are constructed by a simple ligation. Ad-P-EGFP is a recombinant first generation E1-deleted adenovirus where a unique and rare restriction site (*Pac*I) has been engineered at 3.7 mu. The shuttle vector (pC5) also contains a *Pac*I site 3 prime of a CMV-driven expression cassette. pC5 only contains the left ITR and packaging signal from wild-type adenovirus serotype 5, but no regions for homologous recombination. This strategy allows for a much smaller shuttle than usual, making manipulation and subcloning of transgenes easier. Once the gene of interest is cloned in the expression cassette of pC5, the resulting plasmid is digested with *Pac*I and *Swa*I (another rare cutter engineered just in front of the left ITR) to free a fragment containing all the necessary elements for the construction of a recombinant adenovirus (left ITR, packaging signal and expression cassette). Another engineered *Pac*I site in the backbone of pC5 allows for easier purification. This fragment is then ligated overnight to unpurified *Pac*I restricted and dephosphorylated Ad-P-EGFP viral DNA. The mixture is then directly lipofected without further manipulation in 293 cells. Cells are covered with an overlay and, after 5 to 10 days, plaques are screened under a fluorescent microscope and non fluorescent plaques picked and amplified. Typical ratios for fluorescent to non fluorescent plaques are between 1:4 to 1:20. This system allows for rapid generation of recombinant adenoviruses, uses common techniques such as ligation and lipofection, and doesn't need homologous recombination, special bacteria, extraction of large DNA fragments or manipulation of cosmids. This system has allowed us to construct more than 15 different viruses lately, viruses that will be ultimately used in animal models.

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Biopsy-veryfied response of severe lupus nephritis to rituximab (anti-CD20 monoclonal antibody) plus cyclophosphamide after biopsy-documented failure to respond to NIH-protocol cyclophosphamide

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Background: The monoclonal anti-CD20 antibody rituximab (Rituxin, Mabthera) is approved for the treatment of certain B-cell lymphomas. Because of its ability to deplete B lymphocytes the drug may be of benefit in antibody-driven diseases. While treatment of proliferative lupus nephritis with cyclophosphamide-based therapies is succesful in most SLE patients, some have persistently active disease despite such treatment; we currently employ an investigational protocol for the use of rituximab in such patients.

Case report: A female patient with SLE since age 20 underwent renal biopsy at age 31 because of urinary abnormalities. Iohexol clearance at that time was 55 ml/min. She was otherwise asymptomatic. The renal histology showed focal proliferative glomerulonephritis, WHO IIIB, activity index 6/24, chronicity index 6/12. Immunofluorescence revealed 3+ IgG, IgM, and complement in the glomeruli. The patient was given cyclosphophamide and corticosteroids according to the usual NIH protocol for six months. A repeat biopsy after these 6 treatments revealed no improvement, the activity and chronicity scores being largely unchanged. Subsequently, the patient enrolled in the rituximab investigational treatment program.
Treatment: The patient was given 4 weekly infusions with 375 mg/m2 rituximab as well as cyclophosphamide 500 mg/m2 × 2 plus methylprednisolone 1000 mg along with the first and fourth anti-CD20 infusions. Oral prednisolone was maintained at 5 mg/day.
Results: The patient tolerated the treatment well with only minor infusion-related side effects. Clinically she remained asymptomatic. Repeat kidney biopsy 3 months after completion of rituximab treatment revealed SLE nephritis WHO class IB with minimal residual activity (activity index 1/24), and chronicity index 6/12. Immune fluorescence revealed only minimal deposits. Iohexol clearance remains stable at 55 ml/min.
Conclusion: This case provides histopathological documentation of a significant treatment benefit from anti-CD20 plus cyclophosphamide in a patient refractory to cyclophosphamide alone.

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Immunodiagnosis and therapeutic immunosuppression in rheumatoid arthritis with ior t1 (anti-CD6) monoclonal antibody

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CD6 antigen is a type I cell membrane glycoprotein belonging to the scavenger receptor cysteine-rich (SRCR) superfamily group B, predominantly expressed by T cells and a B-cell subset. CD6 binds activated leukocyte cell adhesion molecule (ALCAM), a member of the immunoglobulin superfamily (IgSF). ALCAM is expressed on activated T cells, B cells, monocytes, skin fibroblasts, keratinocytes and rheumatoid arthritis synovium, and mediates homophilic and heterophilic adhesion. CD6-ligand interaction has been implicated in cell adhesion, T-cell maturation and regulation of activation, constituting an uncommon type of protein–protein superfamilies interaction. The ior t1 is a murine IgG2a mAb recognizing a different epitope compared to other anti-CD6 mAbs. It is in a phase II clinical trial (PIICT) for cutaneous T-cell lymphomas treatment. Recently, we reported its intravenous therapeutic effect in a *Psoriasis vulgaris* patient. A PIICT was performed in 18 rheumatoid arthritis patients. Technetium-99m-labeled ior t1 mAb (ior t1-99Tcm) joint uptake and body distribution was assessed by anterior and posterior whole body scans and specific regional imaging. Forty-eight hours apart started a therapeutic dose-finding study based on 7 consecutive daily doses at 0.2 mg/kg, 0.4 mg/kg or 0.8 mg/kg of ior t1 mAb intravenous infusion. Clinical evaluation and laboratory analysis were performed weekly. A rapid ior t1-99Tcm/lymphocytes association and a marked radioactivity uptake form inflamed and silent joints were obtained. From biodistribution studies was estimated that more than 0.5% of the ior t1-99Tcm infusion penetrates into hands and feet with inflamed joints. ior t1 joint-imaging was superior to MDP-99Tcm, used as standard method. ior t1 mAb intravenous infusion induced a dose-dependent therapeutic effect. 0.4 mg/kg was defined as the Optimum Biological Dose, with a long-lasting clinical improvement observed in this group. This treatment reduced the number of tender and swollen joints starting at day 4 of the infusions. Adverse events were dose-depending but controlled by medications. It was not observed deep lymphopenia neither opportunistic infections. This is the first clinical report supporting the relevance of the CD6/CD6-ligand model as a potential target for rheumatoid arthritis immunotherapy. A PI-IICT with a humanized version for iort1 mAb is underway.