

## Meeting abstracts

# Abstracts of the 21st European Workshop for Rheumatology Research

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## Poster Discussion A

### Autoantigens and Autoantibodies in RA

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#### P1

#### Anti-keratin antibodies in juvenile idiopathic arthritis

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We discuss the presence of anti-keratin antibodies (AKA) of the IgG class in patients with defined juvenile idiopathic arthritis (JIA). An indirect immunofluorescence test and rat oesophagus substrate was used for the detection and quantification of AKA antibodies in patients' sera. Overall 33/60 patients with JIA had sera positive for AKA (55 %,  $P = 0,0001$ ) ranging from 1:10 to 1:160 dilutions. Following idiopathic arthritis of childhood classification criteria AKA occurred in 2/7 patients with systemic disease (28,6 %), in 13/30 patients with RF negative polyarthritis (43,3 %,  $P = 0,008$ ) and in 15/18 RF positive polyarthritis (83,3 %,  $P = 0,000002$ ). AKA were also found in a small cohort of patients with oligoarthritis (1/3) and psoriatic arthritis (2/2). AKA positivity occurred in 3/26 healthy controls at a 1:20 dilution. The presence of AKA was correlated as well as with the severity of the disease. Our study revealed that AKA was present overall in 18/29 patients (62%) with severe JIA and in 12/26 patients (46,2 %) with non-severe disease, however this did not reach statistical significance ( $P = 0,18$ ). We also observed that AKA remained positive regardless of disease activity. AKA were detectable in 55,6 % patients with active JIA and in 48,6 % patients in the complete or near remission.

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#### P2

#### The significance of antibodies to cyclic citrullinated peptide, antikeratin antibodies, anti-perinuclear factor, rheumatoid factor isotypes and HLA shared epitope in prediction of erosive disease in early rheumatoid arthritis patients

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**Objectives:** To evaluate a predictive value of autoantibody examinations in development of erosive disease in early rheumatoid arthritis (RA).

**Patients and methods:** One hundred and fourteen patients with disease duration less than 2 years after the onset of symptoms were investigated. Only patients who fulfilled the diagnostic criteria for RA either at the beginning of the disease or during the follow-up period were included. The antibodies to cyclic citrullinated peptide (anti-CCP) (Immunoscan RA, Euro-diagnostica, The Netherlands), IgM, IgA and IgG rheumatoid factors (RF) were measured by ELISA, antikeratin antibodies (AKA) and antiperinuclear factor (APF) were detected by indirect immunofluorescence, and the presence of HLA shared epitope (HLA SE) was detected by PCR with sequence specific primers. Patients were divided into two groups, either with erosive or non-erosive changes present on the hand or/and feet radiographs at the end of 24 months follow-up.

**Results:** Seventy-six (66.7%) patients developed bony erosion, whereas 38 (33.3%) remained without destructive changes. The initial anti-CCP, AKA, APF, IgM RF, IgA RF, IgG RF and HLA SE were positive in 50 %, 46 %, 42%, 54%, 47%, 43% and 67 % in erosive group, and in 19%, 14%, 22%, 30%, 27%, 24% and 65% in non-erosive group, respectively. The significant differences between erosive and non-erosive groups were detected for anti-CCP, AKA, IgM RF and IgA RF. The levels of anti-CCP were significantly higher in erosive early RA group ( $159.1 \pm 224.0$  units) vs. non-erosive one ( $85.8 \pm 164.8$ ). Similarly, patients with erosive disease had significantly higher levels of IgM RF, IgA RF and IgG RF ( $3,1 \pm 2.8$ ;  $2.8 \pm 2.6$ ;  $2.8 \pm 2.6$ ) in comparison with patients without erosions ( $1.9 \pm 2.0$ ;  $1.8 \pm 2.6$ ;  $2.1 \pm 2.8$ ).

**Conclusions:** The data showed that a measurement of anti-CCP, individual isotypes of RFs and to a less extent AKA, could be useful for prediction of disease development in the early cases of RA.

**P3**

**Clinical sensitivity of antibodies against cyclic citrullinated peptide in patients with rheumatoid arthritis**

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The synthetic cyclic citrullinated peptide (CCP) is recognised by rheumatoid arthritis (RA) associated antifilaggrin antibodies, previously determined as antikeratin antibodies or perinuclear factor. Antibodies detected by ELISA using CCP as an antigen (anti-CCP) seem to be of prognostic value in patients with RA.

The objective of our study was to determine the clinical sensitivity of anti-CCP in patients with definite RA (according to ARA diagnostic criteria). RF results were considered when measured in the same serum as anti-CCP.

Sera from 97 RA patients (15 M, 82 F) were tested for anti-CCP in duplicates by Immunoscan RA ELISA (Euro-diagnostica). RF was tested in the same serum in 69/97 patients.

In all 4 assay runs, OD of all calibrators vs. their calculated values completely corresponded to the figure in the manufacturer's analysis certificate. When the units of the lowest calibrator D were calculated by the equation of log calibration curve according to the manufacturer's protocol, they were always about 20% (7-17U) above the defined value (50U). This inconsistency of the curve fitting led to a wrong validation in 26/97 (27%) of the RA samples which OD below the OD of the calibrator D, but with calculated results above the defined cut-off at 50U. Therefore, we calculated results by the equation of 3rd degree polynomial curve which fitted perfectly the measured OD values to the defined units.

Out of 97 RA patients, 56 were anti-CCP positive (58%). From 69 patients simultaneously tested for RF and anti-CCP, both tests were positive in 24/69 (35%) and both negative in 23/69 (33%). Anti-CCP were positive in 15/38 (40%) patients with negative RF. Despite lower anti-CCP positivity rate, 16% of samples in RF neg. group exhibited high anti-CCP values:

| Anti-CCP (U)    | <50 (neg) | 50–200  | 201–800 | 801–3200 | >3200   |
|-----------------|-----------|---------|---------|----------|---------|
| RF pos (n = 31) | 7 (23%)   | 4 (13%) | 6 (19%) | 5 (16%)  | 9 (29%) |
| RF neg (n = 38) | 23 (60%)  | 6 (16%) | 3 (8%)  | 4 (11%)  | 2 (5%)  |

The clinical sensitivity of the anti-CCP test in our RA patients was 58%, which is lower than previously reported (68%). The discrepancy might partially be due to different calculation of the results. We believe that the introduction of polynomial standard curve could contribute to a more consistent validation of samples exhibiting OD below the lowest calibrator.

Our results support the idea that anti-CCP are of diagnostic value especially in RF neg patients.

**P4**

**Expression of citrullin-containing antigens in RA synovium**

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**Introduction:** The presence of autoantibodies directed to citrullinated antigens in serum is highly specific for RA. The aim of this study was to compare anti-CCP concentrations in paired serum/SF

samples of patients with RA and to investigate whether this is associated with the expression of citrullinated antigens in RA synovium and to study the nature of these antigens.

**Methods:** A recombinant single-chain variable fragment (scFv) monoclonal antibody was selected against a cyclic citrullinated peptide (CCP) from a patient antibody-fragment phage-display library. This scFv and patient antibodies affinity purified with CCP, were both used for immunohistochemical staining of synovial cryostat sections of RA (30) and control patients (OA (13), ReA (9), and other arthritides (28)). In addition, rabbit anti-citrullin antibodies (Biogenesis) were used for immunohistochemistry of synovial cryostat sections of RA (14), and control patients (OA (10), ReA (7), and other arthritides (23)). IgG anti CCP titers were calculated with the quantitative Rapsan RA ELISA kit (Eurodiagnostica). Total IgG concentrations were determined on a cobas Fara-2 centrifugal analyzer.

**Results:** Citrullin containing antigens were observed in synovial cryostat sections of anti-CCP positive and negative patients. Staining with ScFv monoclonal antibody was noted in synovial lining cells and in (peri)vascular areas in 13/30 RA patients, 7/13 OA patients, 5/9 ReA patients, and 12/28 other arthritis patients. CCP positivity was on average similar in all diagnostic groups. Staining was absent in the negative controls using a control scFv antibody. Staining with rabbit anti-citrullin polyclonal antibody was noted in 8/14 RA patients, 3/10 OA patients, 2/7 ReA patients, and 6/23 other arthritides. However, controls using irrelevant rabbit antibodies were also positive in some patients in all groups. Anti-CCP concentrations (expressed in Units per mg total IgG) were on average 1.34 times higher in SF compared to serum (n = 20, P < 0.05) or 1.37 when only positive samples were included (n = 11, P < 0.05)

**Conclusion:** Citrullinated antigens are present in the synovia of both RA and control patients with similar prevalence. The presence of anti-CCP autoantibodies in serum is not associated with the expression of citrullinated antigens in the synovium. The identity of the citrullinated antigens and potential differences between RA and control synovia remain to be identified.

**P5**

**Autoreactivity patterns in rheumatoid arthritis**

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Rheumatoid arthritis (RA) is characterized by the occurrence of autoreactive antibodies and T cells. RA is heterogeneous disease also with respect to these autoreactivities, since none of them is present in every RA patient and they are additionally also present – although to a considerably lesser extent - in other autoimmune diseases and even in healthy individuals. It has now been analyzed if there are clusters of autoreactivities that are absolutely specific for RA.

Therefore, the RA-associated autoantigens RA33 (hnRNP A2), citrulline, rheumatoid factor (RF), the stress protein BiP (heavy chain binding protein), calpastatin (Calp) and calreticulin (Calr) have either been biochemically purified or used as a kit of recombinant antigen or chemically synthesized peptides. These antigens have been applied to screen sera and PBMCs from RA and control patients for reactivity and the data have subsequently been subjected to cluster analyses.

Analyzing the reactivities of 100 RA and 100 control patients, the following patterns of the three combined autoreactivities were determined to be absolutely specific for RA: RF+Cit+BiP+, RF-Cit+BiP+. RA-specific patterns composed of four autoreactivities are RA33+RF+Cit+BiP+, RA33-RF+Cit+BiP+, RA33+RF+Cit+BiP-, RA33+RF-Cit+BiP+, RA33+RF+Cit-BiP+, RA33-RF+Cit-BiP+.

RA-specific patterns composed of five autoreactivities are RA33+RF+Cit+BiP+Calp+, RA33-RF+Cit+BiP+Calp+, RA33+RF+Cit+BiP-Calp+, RA33+RF-Cit+BiP+Calp+, RA33+RF+Cit+BiP+Calp-, RA33+RF+Cit+BiP-Calp-. RA-specific autoreactivities composed of six autoreactivities are RA33+RF+Cit+BiP+Calp+Calr+, RA33-RF+Cit+BiP+Calp+Calr+, RA33+RF+Cit+BiP-Calp-Calr+, RA33-RF+Cit+BiP-Calp+Calr+, RA33-RF+Cit+BiP-Calp-Calr-. Other patterns also occurred exclusively in RA patients, but the differences did not reach statistical significance. Patterns including p205-reactivity have yet to be analyzed and will be presented. Summing up the RA-specific patterns that are complementary to each other, the sensitivity of the analysis for RA is 54%.

It appears evident that analyzing other known and unknown RA-associated autoantigens will further increase the sensitivity of the autoreactivity cluster analysis. The diagnostic confidence will markedly improve by testing autoreactivity patterns that clearly distinguish RA from other rheumatic diseases. The composition of the autoreactivity patterns will also improve our understanding of the pathogenesis.

## P6

### Detection of rheumatoid arthritis-specific anti-filaggrin antibodies by line immunoassay shows complementarity to RF and corresponds to the AFA-blot using natural antigen

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Anti-filaggrin autoantibodies (AFA) are highly specific markers for rheumatoid arthritis (RA) and can be detected by immunoblotting using human epidermal protein extracts. Furthermore, it was demonstrated that citrullination of the filaggrin epitopes is crucial for epitope recognition and that citrullinated peptides are also recognized by these specific autoantibodies. Based on these data, a line immunoassay (LIA) was developed containing as individual markers in vitro citrullinated recombinant filaggrin and two citrullinated synthetic peptides.

Firstly, a comparison was made between this prototype LIA and the AFA blot using natural filaggrin. A blind serum panel consisting of 25 early RA, 25 longstanding RA, and 25 disease controls was selected. Results showed a similar performance of both tests at a specificity level of 95%, while the LIA proved significantly better ( $P = 0.035$ ) than the AFA blot at 99% specificity. At the latter specificity level, 2 out of 17 RF negative samples were retrieved on LIA but not on Western blot.

The LIA was further evaluated on sera obtained from 335 RA patients and 254 patients with non-RA rheumatological disorders in a retrospective study. The overall sensitivity of the LIA including three markers (LIA3) was 65.1% versus 61.8% if only the reactivity towards the citrullinated peptides was considered (LIA2). The specificity of LIA3 was 97.6% versus 98.4% for LIA2, which correlates with an estimated positive predictive value (PPV) of 87.3% for LIA3 and 90.7% for LIA2. Thirty-seven percent (30/81) of the RF-negative RA samples proved LIA2-positive, while 52 out of 55 RF positive control samples were negative for anti-filaggrin. Higher specificity and sensitivity was obtained for LIA2 in comparison with anti-RA33 immunoblot, whereas good agreement could be observed with anti-keratin antibody (AKA) testing.

In conclusion, anti-filaggrin autoantibodies can be readily detected by a LIA test based on citrullinated peptides, resulting in a high specificity and hence high PPV for RA. The assay can serve as a user-friendly alternative for AKA immunofluorescent and immunoblot techniques. Together with the RF complementarity, this test provides a valuable tool in the differential diagnosis of RA.

## P7

### ELISA detection of antifilaggrin autoantibodies onto deiminated recombinant rat filaggrin: a highly effective test for the diagnosis of rheumatoid arthritis

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We developed an ELISA using a deiminated recombinant rat filaggrin (ArFA-ELISA) and assessed its diagnostic value for Rheumatoid Arthritis (RA). 714 sera from well characterised rheumatic patients, including 241 RA, were analysed. The results were compared to those obtained with another ELISA using a recombinant filaggrin of human origin and with those of two reference tests.

Recombinant rat filaggrin was obtained by PCR amplification of Wistar rat genomic DNA, cloning, production in *E. Coli* and purification by metal chelate chromatography. The affinity-purified filaggrin was deiminated with type II rabbit skeletal muscle peptidylarginine deiminase. Deiminated and non-deiminated filaggrin were used as immunosorbents and the difference between optical densities on the two antigens were considered as the titer. The other tests were performed following previously described methods : 'AKA' were assayed by semiquantitative indirect immunofluorescence, antibodies to human epidermis filaggrin by immunoblotting (AhFA-IB) and by a recently described ELISA, using a deiminated recombinant human filaggrin (AhFA-ELISA).

Whatever the chosen specificity threshold, the diagnostic sensitivity of ArFA-ELISA was significantly higher than that of the three other tests.

|            | Specificity $\geq 95\%$ | Specificity $\geq 99\%$ |
|------------|-------------------------|-------------------------|
| 'AKA'      | 0.51 (0.45 – 0.58)      | 0.40 (0.34 - 0.46)      |
| AhFA-IB    | 0.59 (0.53 – 0.66)      | 0.37 (0.30 - 0.43)      |
| AhFA-ELISA | 0.52 (0.46 – 0.59)      | 0.31 (0.25 - 0.37)      |
| ArFA-ELISA | 0.75 (0.70 – 0.81)      | 0.61 (0.54 - 0.67)      |

As expected, the titres of ArFA-ELISA, 'AKA', AhFA-IB and AhFA-ELISA were closely correlated ( $P < 10^{-5}$ ). However, among RA sera, only 53% were concordant for the four tests, 25% being positive with only one test. Consequently, the successive use of ArFA-ELISA, then 'AKA' detection only when the first test is negative, would allow a diagnostic sensitivity of 0.67 to be reached, keeping a specificity close to 0.99.

This ArFA-ELISA appears as one of the most efficient among the tests previously described for detection of antifilaggrin/anti-citrullinated peptides autoantibodies, in terms of diagnostic accuracy for RA.

Its diagnostic performance in early RA and its prognostic value are currently under evaluation.

## P8

### Investigation of the epitopes of human profilaggrin derived peptide targeted by antibodies of patient with rheumatoid arthritis

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Anti-filaggrin antibodies (AFA), directed against the 37-40 kD epidermal protein filaggrin are one of the most specific markers of rheumatoid arthritis (RA) and include anti-keratin antibodies (AKA) and anti-perinuclear factor (APF). Although the antigen triggering autoimmune response to filaggrin related proteins has not been identified, recent studies confirmed that citrulline is essential constituent of protein related antigenic determinants recognised by RA specific autoantibody population.

The aim of our study was to identify epitopes of filaggrin derived-peptides targeted by RA specific antibodies to provide further information about the nature of the initial autoantigenic substance.

Citrullin containing peptides of human profilaggrin region (amino acid residues 306-324) derived from known cDNA and on the basis of the data published by Shellekens were synthesised by the multipin peptide synthesis on solid support and were reacted in situ by patient sera. Two 19-mer peptides were prepared with single citrullin substitution at position 312 or 321 respectively and four additional ones with simultaneous replacements of two Arg by Cit. Shortened versions of the <sup>306</sup>SHQESTCitGRSGRSGRSGR<sup>324</sup> peptide were also produced by removal of 1-6 amino acid residues from its N-terminal and the 14-mer truncated one was further shortened from its C-terminal as well. The reactivities of these peptides with RA sera and healthy controls were determined. The results showed that substitution of arginine 312 by citrulline plays major role in the antigenicity of filaggrin-derived sequences. Peptides not containing Cit in position 312 almost lost their ability to bind antibodies from RA sera. Replacement of one additional Arg by Cit in different positions did not improve the antigenicity. When the peptide with Cit in position 312 were shortened from its N-terminal, the 14-mer one showed the highest reactivity. Further shortening of this sequence from its C-terminal showed that TXGRS motif seems to be essential to comprise the autoantigenic epitope.

In conclusion our results provide further evidence that not simply the presence of citrullin but also the nature of its surrounding amino acids have important role in the creation of autoantigenic epitope reactive with anti-filaggrin antibodies.

## P9

### Anti-skin anti-intercellular antibodies in juvenile idiopathic arthritis

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The aim of this work was to study the presence of anti-skin anti-intercellular (ASA-IC) and anti-basement membrane (ASA-BM) antibodies of the IgG class in patients with juvenile idiopathic arthritis (JIA) without clinical features of chronic vesicular-bullous diseases including pemphigus, pemphigoid and epidermolysis bullosa acquisita (EBA).

No D-penicillamine was used for JIA management in this group due to a risk of drug-induced pemphigus. Indirect immunofluorescence antibody test (IIF) and dual substrates of monkey and guinea pig esophagus sections were used for the detection and quantification of ASA-IC as well as ASA-BM antibodies. Overall ASA-IC were detected in 50 out of 57 studied patients' sera samples (87,7 %,  $P = 0,0003$ ) ranging from 1:20 to  $\leq 1:320$  dilutions. Respective of the classification criteria for idiopathic arthritis of childhood ASA-IC were observed in 6/6 patients with systemic disease (100%,  $P = 0,029$ ), 24/29 patients with RF negative polyarthritis (82,7 %,  $P = 0,01$ ), 16/18 RF positive polyarthritis (88,9 %,  $P = 0,0077$ ) as well as in a small cohort of patients with oligoarthritis (2/2) and psoriatic arthritis (2/2). However we have observed a high incidence of anti-skin anti-intercellular antibodies in a cohort of patients with JIA we suggest that subclinical pemphigus occurring in this group might be exacerbated with different stimulus including pemphigus inducing drugs. No ASA-BM antibody positivity was observed in a cohort of 57 studied patients.

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## P10

### Correlation of the humoral immune answer to selected bacterial antigens with presence of the DNA specific to *Salmonella enteritidis* after amplification by PCR

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**Introduction:** An infectious aetiology has often been discussed as a most compatible with both the clinical and pathological features of rheumatoid arthritis (RA). Until now, no single microorganism can be shown as consistently associated with development of RA. In our former serological and molecular studies we have shown that the most common humoral immune answer in RA patients is directed to *Salmonella enteritidis* /S. ent./ antigens, especially to specific for *Salmonella enteritidis* O3 LPS.

The aim of the study was to prove the correlation between systemic and local humoral immune answer to *Salmonella enteritidis* antigens and the presence of DNA specific for *Salmonella enteritidis* O3 serotype.

**Materials and methods:** In the tested group, composed of 35 sera and 20 synovial fluid, taken from 20 patients with connective tissue diseases the presence of DNA after PCR amplification and antibodies by ELISA method were estimated.

**Results:** In 10 of 35 (31%) synovial fluids /bacteriologically negative/ we have found /after amplification by PCR/ double band of the DNA, specific for *Salmonella enteritidis*, possessing mol. weight 390bp and 420bp respectively. Also in the same group of patients the antibodies to OMP S. ent. in 30% of tested cases, to LPS S. ent. in 78,9%, in 30% to ECA and none to peptidoglycan have been found. Only in a few of the PCR-positive synovial fluid elevated level of antibodies to S. ent. have been found.

**Conclusions:** No evident correlation, so far, between class and specificity of humoral antibodies and the presence of specific for S. ent. DNA after PCR amplification have been found.

**P11****Aberrant expression of the autoantigen hnRNP-A2/RA33 in the joints of patients with rheumatoid arthritis and TNF-alpha transgenic mice: a clue to autoimmunity?****S Hayer, M Tohidast-Akrad, G Schett, K Skriner, D Plows, S Haralambos, G Kollias, J Smolen and G Steiner***Division of Rheumatology, University of Vienna; L. Boltzmann Institute of Rheumatology, Vienna, Austria; Institut Pasteur Hellenique, Athens, Greece.*

Autoantibodies to the nuclear antigen hnRNP-A2/RA33 are present in sera of RA patients and also in TNF $\alpha$  transgenic (tg) mice which develop severe erosive arthritis similar to human disease. Furthermore, autoreactive T cells have recently been found in peripheral blood of 60% of RA patients suggesting hnRNP-A2/RA33 to be also a major T cell autoantigen.

To investigate the pathway leading to loss of tolerance, expression of hnRNP-A2/RA33 was investigated by immunohistochemistry in synovial tissue derived from patients with RA and osteoarthritis (OA) as well as in joint sections of TNF $\alpha$  tg and control mice. These analyses revealed the antigen to be considerably overexpressed in RA as compared to OA tissue, particularly in macrophages of the lining layer and in fibroblasts of the sublining areas, whereas no or very little expression was observed in areas of lymphocyte infiltration. Remarkably, the antigen was not only located in the nucleus (as in cultured cells) but also abundantly detectable in the cytoplasm. Similar observations were made in TNF $\alpha$  tg animals in which strong hnRNP-A2/RA33 expression could be also observed at cartilage-pannus junctions. Interestingly, anti-A2/RA33 aab were not detectable in 8 animals treated with tissue inhibitor of metalloproteases 1 (TIMP 1) suggesting that metalloproteases may be involved in breakage of tolerance to hnRNP-A2/RA33. To elucidate the biological mechanisms leading to aberrant expression of hnRNP-A2/RA33 peripheral blood monocytes and T lymphocytes as well as cultured synovial fibroblasts and HeLa cells were treated with various stimulating agents including LPS, PHA, anti-CD3, IL-1, TNF $\alpha$  and IFN $\gamma$  or exposed to heat stress. However, none of these stimuli was able to induce upregulation or nucleocytoplasmic translocation of the antigen.

Taken together, these findings suggest that the state of chronic inflammation in the rheumatoid synovium causes aberrant expression of hnRNP-A2/RA33 (and possibly other autoantigens). This may lead to increased uptake and (aberrant) degradation and presentation by macrophages and other antigen presenting cells subsequently activating autoreactive T cells which then may drive or enhance the inflammatory and destructive processes characteristic of RA.

**P12****Autoantibodies to the mRNA destabilizing protein hnRNP-D/AUF1 in patients with systemic autoimmune diseases****K Skriner, W Hueber, E Süleymanoglu, E Höfler, JS Smolen and G Steiner***Division of Rheumatology and Institute of Biochemistry, University of Vienna, 2nd Department of Medicine, Lainz Hospital, Vienna, Austria*

The heterogeneous nuclear ribonucleoprotein (hnRNP) D, which is also known as AU-rich element binding factor 1 (AUF1), decreases stability of many short-lived mRNAs (including mRNAs of IL-1 and TNF $\alpha$ ) by binding to adenosine and uridine rich sequences (ARE) contained in their 3'-untranslated regions. Previous studies had indicated this protein to be recognized by sera from patients RA, SLE and MCTD.

To investigate this autoimmune response in greater detail 356 sera from patients (pts) with various rheumatic disorders were tested by immunoblotting employing the recombinant 45 kDa variant of D/AUF1 (the largest of the 4 known D/AUF1 proteins). Autoantibodies (aab) were detected in 20% of RA pts ( $n = 105$ ), 34% of SLE pts ( $n = 70$ ), 17% of MCTD pts ( $n = 31$ ) and in 25% of pts with primary Sjogren's syndrome ( $n = 21$ ), but in less than 5% of 129 pts with other rheumatic disorders and not at all in healthy controls. Importantly, anti-D/AUF1 aab were already present in 25% of 60 patients with early RA of less than 6 months duration, whereas only one of 40 non-RA patients with other forms of early arthritis showed this antibody. Epitope mapping studies showed the aab to be directed to conformational epitopes in the N-terminal part of hnRNP-D/AUF1 known to be indispensable for the protein's function. However, aab did not interfere with RNA binding as assessed by gel shift assays employing the ARE of the TNF $\alpha$  mRNA. Instead, they were able to supershift protein-RNA complexes indicating binding sites for RNA and aab to be distinct.

Thus, these findings suggest that anti-D/AUF1 aab target the mRNA decay complex which may form another large ribonucleoprotein target structure in systemic autoimmunity. We are tempted to speculate that increased formation of such complexes (e.g. due to overexpression of instable mRNAs such as those for IL-1 and TNF $\alpha$  as seen in RA) may lead to pathologic autoimmune reactions against D/AUF1 and other proteins of mRNA decay complexes.

**P13****The stress protein BiP is a major autoantigen in rheumatoid arthritis****U Neuhaus-Steinmetz\*, A Union†, J Raymackers‡, F Schumann\*, S Behrens\*, W Schmid‡, JM Engel§, GR Burmester\* and S Bläß\****\*Department of Rheumatology & Clinical Immunology, Charité University Hospital, Berlin, Germany; †Innogenetics N.V., Ghent, Belgium; ‡Clinic for Rheumatology, Berlin-Buch, Germany; §Clinic for Rheumatology, Bad Liebenwerda, Germany*

BiP (heavy chain binding protein) is the major chaperone of the endoplasmic reticulum that interacts transiently with most of the proteins of the secretory pathway and assists in their folding. BiP's function under stress conditions is essential for cell viability and constitutively increasing or decreasing the BiP levels is detrimental to cell growth or to survival. Here, we present the RA autoantigen formerly designated "p68" as identical to BiP and that autoreactivity against BiP is a specific feature of RA.

p68 was isolated and proven to be identical to BiP by two different approaches (Edman sequencing and MALDI-TOF mass spectrometry). Using tissue sections, BiP has been shown to be overexpressed in the RA as compared to the OA joint. Applying immunoblots, BiP-reactive autoantibodies were present in 63% of 400 RA patients, in 7% of 200 patients with other rheumatic diseases and in 1 of 150 healthy individuals. In patients with early arthritis approximately 50% are positive. An ELISA was established to quantify anti-BiP antibodies and the data of 400 RA and 400 control patients will be presented. The majority of RA sera was found reactive with a posttranslationally modified form of BiP and the major epitope was O-linked N-acetylglucosamine.

Furthermore, we present evidence that BiP-specific T cell reactivity is pathogenically altered in RA. Overt BiP-specific T cell reactivity as determined by T cell proliferation assays could be observed in two thirds of patients with RA, but neither in healthy individuals nor in patients with other rheumatic diseases. Blocking anti-HLA-DR antibodies expectedly decreased T cell proliferation indicating the presence of HLA-DR restricted effector T cells.

A subset of RA patients exhibited a BiP-specifically suppressed T cell reactivity. Blocking anti-HLA-DR antibodies in these assays

overcame the suppressive effect and allowed an increased proliferation. This argues strongly for the presence of BiP-specific regulatory T cells restricted for HLA-DR and BiP-specific effector T cells restricted for HLA-DP and -DQ in this subset of RA patients. These effects could not be mimicked by blocking anti-IL-10 or anti-TGF- $\beta$  antibodies, implicating that other factors or also direct cell-cell contact are required.

Apparently, the healthy immune system views BiP as a component to which autoreactivity is either avoided or tightly regulated. In RA this general principle appears to have lost control. BiP-reactive may serve as a new diagnostic marker in RA, while regulatory T cells may provide means for a specific therapy.

#### P14

### Lysozyme and its biological value in rheumatoid arthritis (RA)

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Lysozyme or muramidase catalyzes the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan. A basic enzyme that is present in saliva, tears, egg white and many animal fluids. Its function is an antibacterial agent. Lysozyme is well known for the ability to hydrolyze the cell wall of bacteria.

**Objective:** The aim of study was to measure the concentration of lysozyme in synovial fluid in RA patients.

**Methods:** We measured the lytic activity of lysozyme towards *micrococcus lysodeikticus* (1, 2, 3), bacteria which are highly susceptible to lysis by lysozyme by the turbidometric method 30 synovial fluid of RA patients. In order to obtain a method covering a wider range of lysozyme concentrations, Osserman and Lawlor worked out the so-called lyso-plate method (4).

The test measured the zone of clearing by lysozyme in an agar plate, in which *micrococcus lysodeikticus* is embedded. After about 18 hours the diameter of the zone of clearing is measured.

**Results:** In all our RA synovial fluid we observed increased level of lysozyme.

**Conclusions:** The increased levels of lysozyme in synovial fluid in RA could indicate of monocyte/macrophage activity and might be used to study disease activity in RA.

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## Poster Discussion B

### Cytokines

#### P15

### Differential effect of corticosteroid therapy on the cytoplasmic cytokine expression in CD4 and CD8 positive T cells from lupus patients

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Due to their different antiinflammatory and immunomodulatory effects corticosteroids are widely used in the treatment of SLE. Literature data support both Th1 and Th2 dominance in lupus. There are only few reports about cytokine profile of CD8+ T cells in SLE.

In the present study we examined by flow cytometry the cytoplasmic IFN $\gamma$ , IL-4 and IL-10 expression in CD4+ and CD8+ T cells from six active, untreated, newly diagnosed SLE patients, who received after that 1 mg/kg methylprednisolon. Pretreatment expression of IFN $\gamma$  was lower, while IL-4 and IL-10 expressions were higher in CD4+, but not in CD8+ T cells of patients than in control cells. Corticosteroid treatment increased IFN $\gamma$  and decreased IL-4 and IL-10 expressions in patients' CD4+ cells, but had no significant effect on the cytoplasmic cytokine expression of CD8+ cells.

In conclusion, present results indicate that corticosteroid therapy does not influence IFN $\gamma$ , IL-4 and IL-10 expression in CD8+ T cells of lupus patients, and it may have pathogenic significance.

#### P16

### Elevated IL-16 level is a non-genetic characteristic of patients with severe systemic lupus erythematosus

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**Introduction:** IL-16, originally named lymphocyte chemoattractant factor, is a cytokine which is mainly produced by CD8+ T cells. Several reports have described that increased levels of IL-16 are in part responsible for T cell abnormalities in SLE patients. It is unknown if the previously reported increased levels of IL-16 is a characteristic underlying susceptibility to SLE or is a characteristic of the disease itself.

**Methods:** Accumulated organ damage was measured with the SLICC/ACR Damage Index. Twenty-five severe (SLICC/ACR: 4.9  $\pm$  2.5) and ten non-severe (SLICC/ACR: 1.0  $\pm$  0.8) SLE patients were included in this study. Also 11 first degree relatives and 12 healthy volunteers were included in this study. Plasma IL-16 levels were measured by ELISA.

**Results:** No significant difference in the IL-16 levels of the first degree relatives of patients with SLE (38.3  $\pm$  11.1 pg/ml) were observed when compared to controls (31.2  $\pm$  10.1 pg/ml). In order to analyze characteristics of the SLE in relation to concentration of IL-16, IL-16 was measured in severe SLE patients (71.3  $\pm$  87.4 pg/ml;  $P = 0.025$ ) compared to healthy controls. On the other hand, no significant differences were observed between the non-severe SLE patients (37.8  $\pm$  26.1 pg/ml) and controls.

**Conclusion:** No evidence for increased IL-16 levels in first degree relatives of the SLE patients was observed. IL-16 is enhanced in SLE patients with a severe disease, but not in patients with non-severe disease, thereby suggesting that IL-16 is associated with disease severity, and not with susceptibility for SLE.

**P17****Functional and biologic evaluation of immunoregulatory cytokines in the immunopathologic lesion of Sjogren's syndrome****DI Mitsias, AG Tzioufas, CI Veiopoulou, HM Moutsopoulos, G Thyphronitis***Department of Pathophysiology, Medical School, University of Athens*

Autoimmune diseases are commonly considered to involve a Th1/Th2 imbalance in favor of Th1. Studies in mice suggest that the Th1 cytokines overexpression may be implicated in the pathogenesis of autoimmunity. Recent results, though, in humans challenge this notion.

Conflicting data have been published on the cytokine profile in patients with Sjogren's syndrome (S.s.). These data were mainly obtained using PCR based techniques and in-situ hybridization. Some of them indicate that a Th1 response is prevalent. To clarify this issue, small salivary glands (SGL) from patients with S.s. with different grades of infiltration as well as patients without S.s. but with sicca manifestations, were cultured in RPMI plus 40 IU/ml of recombinant IL2. IL4, IL13 and IFN $\gamma$  production in the culture supernatants (SN) was evaluated with a sensitive ELISA and, in parallel, mRNA levels for the same cytokines were evaluated with a quantitative RT-PCR.

Within a period of a week, lymphocytes were evident in the cultures in numbers depending on the infiltration of the gland. The phenotype of these lymphocytes, as determined by flow cytometry, was similar to that published previously from SGL immunochemistry, suggesting that these cells derive from the salivary gland. SN were collected on various time intervals and mRNA was extracted from the lymphocytes on day 12 of the culture. Interestingly, IL13 mRNA was detected in almost all (17/19) of the samples, and both IFN $\gamma$  and IL4 on the majority (16/19 and 14/19, respectively). The cytokine production in the culture SN was examined in a set of 8 biopsies. IL 4 couldn't be detected (<4 pg/ml), may be due to its binding on the IL4R. Both IFN $\gamma$  and IL13 were present and the ratio of IFN $\gamma$ /IL13 was related to the grade of infiltration ( $p < 0.05$ ), indicating that the balance of Th1 vs Th2 changes in favor of the former along with the severity of the disease. Finally, it was demonstrated that the Th2 cytokines IL4 and IL13 are functional since IgE was present in 12 out of 28 SN of cultured biopsies. IgE was present in greater quantities in those biopsies that had low grade infiltration (0-0.3 according to Chisholm's score) compared to those with medium grade (0.3-0.6), while in biopsies with infiltration >0.6 it was undetectable.

These results show that in Sjogren's syndrome the distinction between Th1 and Th2 doesn't apply as both play a role to its pathogenesis. Moreover it seems that Th2 response prevails at the early stages whereas Th1 gradually augments as the disease progresses.

**P18****High spontaneous CD40 expression by salivary gland epithelial cells in Sjogren's syndrome: possible evidence for intrinsic activation of epithelial cells****ID Dimitriou, G Xanthou, EK Kapsogeorgou, RF Abu-Helu, HM Moutsopoulos and MN Manoussakis***Department of Pathophysiology, Medical School, University of Athens, Greece*

CD40 is a surface protein originally identified on B cells. Its interaction with CD40L on T cells plays an important role in the activation, proliferation and differentiation of B cells. During the recent years,

CD40 has been identified in an expanding list of hemopoietic and nonhemopoietic cells and has received an increased interest based on its role in a variety of cell-mediated responses and its potential to participate in the pathogenesis of chronic inflammatory disorders. Sjogren's Syndrome (SS) is an autoimmune exocrinopathy, which is characterized by chronic lymphocytic infiltration of exocrine glands and aberrant activation of epithelial tissues.

To investigate the participation of CD40 in the pathogenesis of SS, the expression of this protein was studied in cultured non-neoplastic salivary gland (SG) epithelial cell (SGEC) lines as well as in minor SG biopsies obtained from 17 SS patients and 12 controls. Immunocytochemical and flow cytometric analysis has revealed the occurrence of constitutively expressed CD40 molecules on the surface of our long-term cultured SGEC lines, which could be further induced by IFN $\gamma$  and IL-1 $\beta$ , but not TNF $\alpha$ , IL-4, IL-6, GM-CSF and IFN $\gamma$ . In SGEC lines derived from SS patients, the spontaneous expression of membranous CD40 was significantly higher compared to controls ( $P < 0,001$ ), which is likely suggestive of their activated status. In SG biopsies, CD40 was constitutively expressed by B cells, ductal epithelial cells and endothelial cells but not by other glandular cell types, such as acinar cells, myoepithelial cells and fibroblasts. In addition, CD40L staining was also detected in 30-50% of the infiltrating T cells in the biopsies of SS patients.

Our results possibly reveal the immunoregulatory potential of SGEC and lend further support to a model of intrinsic activation in salivary epithelia in SS, whereby these cells actively participate in the induction and maintenance of lymphocytic infiltrates of patients.

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**P19****Substance P and its cleavage products: effects on interleukin-1 secretion of rheumatoid arthritis monocytes/macrophages****E Wagner, G Partsch\* and A Dunky***5th Medical Department, Wilhelminenspital, Vienna, Austria; \*Ludwig Boltzmann Institute of Rheumatology and Balneology, Vienna, Oberlaa, Austria*

**Introduction:** Clinical observations in man and experimental studies support the importance of neurogenic mechanisms in the initiation and perpetuation of inflammation. A leading role is attributed to substance P (SP), a tachykinin that besides its role in pain transmission in the central nervous system is also secreted antidromically from peripheral nerve endings into tissue in response to various stimuli. Since SP might be cleaved into several cleavage products (CLP) in the synovial fluid, we determined the effects of SP and its CLPs on the production of IL-1 by monocytes/macrophages (MO) from patients with rheumatoid arthritis.

**Methods:** MO from venous blood were separated and stimulated with SP and its CLPs in various concentrations without and with LPS stimulation (1 $\mu$ g/ml). The IL-1 concentration in the supernatant was determined by ELISA. The CLPs SP 1-4, SP 4-11, SP 6-11, SP 7-11 were applied in the experiments.

**Results:** SP and its CLPs (without LPS) had only weak and low (in pM range) effects on IL-1 secretion. The stimulating effect of SP and SP 7-11 was pronounced at lower concentrations, at higher concentrations inhibition was observed. The other CLPs did not show significant effects.

The same result was observed in MO with prior LPS stimulation. However, with LPS there was an increase of IL-1 in the nMolar range.

**Discussion:** In contrast to the findings of other authors SP and SP 7-11 at higher concentrations inhibited IL-1 release from MO. The effects were similar in LPS stimulated and LPS unstimulated MO, therefore not due to full LPS stimulation at the concentration of

1 µg/ml. This could point to a mechanism of action different from the classical neurokinin receptor of SP and CLPs on MO regarding cytokine secretion.

## P20

### Human neutrophil production and cleavage of IL-18: potentiating inflammatory arthritis?

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We have recently demonstrated the presence and involvement of IL-18 in rheumatoid arthritis (RA) synovitis. Moreover, blockade of IL-18 *in vivo* is protective in arthritis models. We sought to demonstrate for the first time the production and intracellular processing of IL-18 by human neutrophils. Thereafter we investigated novel processing pathways and potential regulatory mechanisms for IL-18 bioactivity that could operate in synovium.

**Methods:** Matched peripheral blood (PB) and synovial fluid (SF) neutrophils were isolated by ficoll density gradients. IL-18 was detected in neutrophil total protein lysates by western blotting. Serum free neutrophil culture supernatants were incubated with recombinant IL-18 prior to HPLC fractionation and assessed for biological activity using IL-18 sensitive KG-1 cells.

**Results:** Western blotting of neutrophil lysates isolated from PB and SF of rheumatoid and psoriatic arthritis patients demonstrated the presence of a number of IL-18 specific bands ranging in molecular weight from 22 to 6 kD in size, representing pro, mature and possible IL-18 cleavage products. HPLC purified culture supernatants from PB and SF neutrophils contain heat sensitive enzymatic activity capable of *in vitro* cleavage of both recombinant pro and mature IL-18. This caspase independent cleavage of IL-18 resulted in the generation of biologically active fragments capable of modulating IL-18 induced IFN- $\gamma$  production by KG-1 cells.

**Conclusions:** These data demonstrate for the first time that modified fractions of IL-18 may be biologically active, suggesting the existence of a novel regulatory mechanism in the IL-1 cytokine family. In light of their rapid accumulation in large numbers within RA joints, our data further suggest that both neutrophils and IL-18 play important roles in disease pathogenesis.

## P21

### Intracellular expression of CXCR3 on rheumatoid arthritis synovial tissue cells

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**Introduction:** Inflammatory cell infiltration and synovial activation are important processes in rheumatoid arthritis. Chemotactic gradients of various chemokines are responsible for cell attraction and possibly for their activation. We have previously detected strong expression of chemokine receptor CXCR3 in the rheumatoid joint by immunostaining.

**Aim:** Characterization of the cells expressing CXCR3 in RA synovial membrane.

**Methods:** Synovial tissue samples were obtained from RA patients undergoing 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 mouse MAb (R&D) and with a panel of phycoerythrin (PE) conjugated MAb (anti-CD3, CD4, CD8, CD19, CD55, CD31, CD68, CD14 and CD45). Live cells were identified by propidium iodide. PBCs were stained using the same protocol.

**Results:** As expected, a proportion of CD3+ and CD4+ blood and synovial cells were CXCR3 positive. In addition, CXCR3 was also seen in synovial cells positive for CD55, CD14, CD8 and to a lesser extent CD31. However, in contrast to the surface staining of cells from peripheral blood, synovial cells displayed only intracellular staining for CXCR3. No CXCR3 staining could be detected on the surface of any type of viable synovial cell, including CD3 positive lymphocytes.

**Conclusions:** Flow cytometry identifies synovial cells that display intracellular CXCR3 staining. These cells are comprised of T lymphocytes, macrophages, possibly synovial fibroblasts and endothelial cell populations. The intracellular presence of CXCR3 suggests a possible internalization of this molecule, which may be a consequence of ligand binding. The significance of this phenomenon and of CXCR3 expression in cell types other than leukocytes remains to be determined.

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## P22

### Interleukin-13 (IL-13) in autoimmune rheumatic diseases: relationship with autoantibody profile

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The production of rheumatoid factor (RF) and antinuclear antibody by B-cells could depend on different cytokines action. We evaluated IL-13 serum levels in 230 patients with autoimmune rheumatic diseases including rheumatoid arthritis (RA) [M/F=22/62; mean age=55.2 (25-76) yrs; mean disease duration = 116 (5-605) months], SLE [M/F=17/97; mean age=38.3 (15-70) yrs; mean disease duration = 77 (1-456) months], Sjögren's syndrome (SS) [M/F=2/50; mean age = 55.2 (26-81) yrs; mean disease duration = 82 (3-540) months], and systemic sclerosis (ScS) [M/F = 1/31; mean age=50.6 (20-73) yrs; mean disease duration = 113 (12-276) months], in order to investigate the relationship of this cytokine with the autoantibody profile.

Serum levels of IL-13 (pg/ml) were significantly increased in patients with RA ( $P < 0.00003$ ), with SLE ( $P < 0.03$ ), with SS ( $P < 0.0007$ ), with ScS ( $P < 0.025$ ) as compared to controls. IL-13 serum levels correlated with those of RF in RA ( $P < 0.00001$ ), SLE ( $P < 0.003$ ) and ScS ( $P < 0.03$ ). IL-13 levels were higher in RA ( $P < 0.0002$ ), SLE ( $P < 0.004$ ) and ScS ( $P < 0.05$ ) patients with RF than patients without RF. SS patients with anti-SSA/Ro antibodies had significantly higher IL-13 levels than SS patients without this autoantibody ( $P < 0.036$ ). No statistically significant correlation was found between IL-13 levels and any other antinuclear autoantibody or total immunoglobulin levels or main clinical features of each disease.

This study suggests that IL-13 may be involved in the pathogenesis of autoimmune rheumatic diseases, with a relevant role on RF production. In SS, the lack of correlation between IL-13 and RF is probably due to the peculiar characteristics of this antibody in the disease. We can conclude that the mechanisms involved in RF synthesis recognise different pathways depending on the underlying autoimmune disease.



**P23****Mice deficient in leptin (ob/ob) or in leptin receptor (db/db) have a milder form of antigen-induced arthritis****N Busso, A So, V Péclat and C Gabay\****Service de Rhumatologie, CHUV, 1100 Lausanne, Switzerland;  
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Leptin, the product of the ob gene, is synthesized exclusively by adipocytes to regulate the body weight in a central manner through its interaction with long isoform of leptin receptor ob-Rb. However, Ob-Rb is also expressed in lymphoid tissues and leptin has been shown to play an important role in cell-mediated immunity. We therefore decided to examine the role of leptin *in vivo* by analyzing the phenotype of mice deficient in leptin (ob/ob) or in ob-Rb (db/db) during antigen-induced arthritis (AIA). Arthritis was induced by an intraarticular injection of methylated bovine serum albumin (mBSA) in the knees of previously immunized ob/ob, db/db, control littermates and wild-type mice, all in C57BL/6 background. The severity of arthritis was determined by 99m Technetium (99m Tc) uptake. In addition, the degree of articular inflammation was also determined after sacrifice by histology scoring. Levels of circulating immunoglobulins and antibodies against mBSA were measured by ELISA. The responses of isolated lymph node cells to mBSA were also examined. The results showed that joint inflammation, as measured by 99m Tc uptake, was significantly reduced in ob/ob mice as compared with control littermates and wild-type mice (on day 1 of arthritis:  $P < 0.002$  and  $P < 0.001$ , respectively; on day 3:  $P < 0.03$  and  $P < 0.02$ , respectively). In addition, histology studies showed that ob/ob mice had markedly less synovial inflammation than lean controls ( $P < 0.04$ ). In contrast, there was no difference in proteoglycan content within the articular cartilage as assessed by Safranin-O staining. The *in vivo* production of antibodies against mBSA was significantly decreased in ob/ob mice as compared with controls ( $P < 0.03$ ). Circulating levels of IgG2a were also significantly lower in ob/ob mice than in controls, whereas levels of IgM were not different. *In vitro* lymph node cell proliferation in response to mBSA was significantly reduced in ob/ob mice as compared with controls. In addition, production of interferon- $\gamma$  by cultured lymph node cells was significantly lower in ob/ob than in control mice, whereas opposite results were observed for IL-10. Experiments performed in db/db mice confirmed the findings in leptin-deficient mice. In conclusion, leptin appears to regulate both the cellular and humoral components of the immune response against mBSA and to contribute to the mechanisms of joint inflammation in AIA. In addition, these results demonstrate that the effects of leptin on the immune system are mediated through its interaction with ob-Rb.

**P24****Temporal expression of cytokines and chemokines in rat adjuvant-induced arthritis****Z Szekanecz, MM Halloran, JM Woods, MV Volin, GK Haines and AE Koch***Third Department of Medicine, University of Debrecen, Hungary and Northwestern University, Chicago, Illinois, USA*

Adjuvant-induced arthritis (AIA) in rats is a relevant model for human rheumatoid arthritis (RA). In this study, the expression of the cytokines TNF- $\alpha$ , IL-1 and IL-6, as well as the chemokines MIP-1- $\alpha$ , MCP-1 and ENA-78 in the sera and joint homogenates of AIA and control, sham-injected rats was studied over a 47-day period. All of these cytokines and chemokines showed increased production in AIA. In addition, TNF- $\alpha$ , IL-1, ENA-78 and MIP-1- $\alpha$  could be termed as "early" mediators, as their production increased in the first

14-21 days and it correlated with early events in synovitis, such as neutrophil ingress, joint swelling and general symptoms. TNF- $\alpha$  may have mostly systemic, while IL-1 mainly local synovial effects. IL-6 and MCP-1 were found to be "late" inflammatory mediators, as their secretion was up-regulated after 2 weeks post-adjuvant injection and remained high during the observation period. Also, significant correlation was found between the production of TNF- $\alpha$  and that of chemokines. In conclusion, the differential expression of "early" and "late" cytokines and chemokines may account for various events underlying synovitis in AIA.

**P25****Dynamics of early synovial cytokine expression in rodent collagen-induced arthritis: a therapeutic study****K Palmblad\*, H Erlandsson Harris\*, KJ Tracey† and U Andersson\****\*Rheumatology research unit, Karolinska Hospital, CMM L8:04, 171 76 Stockholm, Sweden; †North Shore University Hospital, Manhasset, NY, USA*

This study was performed to elucidate pathophysiological events prior and during the course of collagen-induced arthritis (CIA) in DA rats. Kinetic studies of local cytokine responses were determined using immunohistochemistry and computer-aided image analysis. We also investigated the effect of the macrophage-pacifying compound CNI-1493 on proinflammatory cytokine expressions. Synovial cryosections were analysed at various time points for the presence of IL-1 $\beta$ , TNF and TGF- $\beta$ . Unexpectedly, an early simultaneous TNF and IL-1 $\beta$  expression was detected in resident cells in the lining layer, preceding disease onset by more than one week. The predominant cytokine synthesis by synovial (ED-1+) macrophages coincided with clinical disease. TNF-production greatly exceeded that of IL-1 $\beta$ . CNI-1493 treatment did not affect the early TNF and IL-1 $\beta$  synthesis, while disease-associated TNF and IL-1 $\beta$  production was greatly reduced. Furthermore, CNI-1493 significantly up-regulated synthesis of the anti-inflammatory cytokine TGF- $\beta$  and thereby shifted the balance of pro-inflammatory and anti-inflammatory cytokines in the arthritic joint in a beneficial way.

**P26****Comparison of arthritogenic and nonarthritogenic *Eubacterium aerofaciens* cell walls****X Zhang, M Rimpiläinen, E Simelyte and P Toivanen***Department of Medical Microbiology, Turku Immunology Center, University of Turku, Turku, Finland*

We have recently reported that cell walls (CWs) of two closely related *E. aerofaciens* strains appear arthritogenic or nonarthritogenic when injected *i.p.* into the rats (Zhang et al. Rheumatology 2000). These strains have different structures of the CW peptidoglycan (PG). To further define what determines the arthritogenicity of these human intestinal bacteria, the tissue distribution of their CWs was compared. Muramic acid (MurNAc), a component of PG, was selected as a marker for bacterial CW as it is not synthesized by eukaryotic cells. Gas chromatography-mass spectrometry was applied to identify and quantify MurNAc. The results obtained indicate that the amount of MurNAc was much higher in the spleen and liver after injection of the arthritogenic CW than after injection of the nonarthritogenic CW. MurNAc was detected in synovial tissues and fluids from day 1 to day 28 after injection of the arthritogenic CW, but not after injection of the nonarthritogenic CW. This is probably due to the resistance of the arthritogenic CW against biodegradation; lysozyme and mutanolysin degraded the

arthritogenic CW only by 21-34%, whereas the nonarthritogenic CW was degraded by 77-78%, both after 24h incubation. Furthermore, degradation by mutanolysin significantly increased the capacity of the arthritogenic PG to stimulate rat macrophages to secrete TNF- $\alpha$  and MCP-1, whereas it dramatically decreased such a capacity of the nonarthritogenic PG, suggesting that peptides with proinflammatory activity are released from the arthritogenic PG. These results, obtained with an arthritogenic and nonarthritogenic strains of *E. aerofaciens*, indicate that capacity to resist biodegradation, leading to persistence in the tissues, and to release proinflammatory PG peptides, are crucial factors determining arthritogenicity or nonarthritogenicity of a bacterial CW.

## P27

### Immune complex stimulation of peripheral blood mononuclear cells result in enhancement of suppression of IL-12 production dependent on soluble serum factors

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Immune complexes can induce the production of various cytokines in vitro. Both IL-10 and IL-12 could be induced by addition of heat-aggregated immunoglobulins to mononuclear cells in serum-free cell culture systems. Addition of native serum to the cell cultures influenced the effects on IL-10 and IL-12 in opposite ways. While IL-10 levels were increased in cell cultures with native human serum, IL-12 production was inhibited as compared to cultures with monomeric IgG. Two series of experiments suggested that the effects of immune complexes on IL-12 production depended on the activity of the classical complement pathway in the serum: 1.) Heat-inactivation of serum reverted the inhibitory effect of immune complexes on IL-12 production. 2.) C4 deficient serum behaved as a heat-inactivated normal serum concerning the effects on IL-12 production, and this effect could be reversed by addition of C4. The effects of neutralizing IL-12 had modest effect on immune complex-induced IL-10 production, and the effects of neutralizing IL-10 had no effect on IL-12 production. IL-10 production in the presence of immune complexes could be partially blocked by anti-Fc $\gamma$ R2 antibodies, while the immune complex-mediated effects on IL-12 not changed by blocking Fc $\gamma$ R2 or Fc $\gamma$ R1. Opposite and complement-dependent effects on the production of IL-10 and IL-12 can be of importance in cytokine-dependent autoimmune diseases like rheumatoid arthritis or systemic lupus erythematosus, where local or systemic activation of the classical complement pathway participate in the disease processes. Blocking of complement activation or receptors for activated complement components might gain increased attention as potential targets for immune therapies in the light of such cytokine-deviating effects.

## P28

### Inflammatory arthritis, hypoxia and vascularity

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We have employed novel technology to investigate the relationship between synovial tissue oxygen levels and vascularity in human inflammatory arthritis. Silver microelectrodes were used to measure

synovial tissue oxygen levels in knee joints of 15 patients with inflammatory arthritis (6 RA, 2 SLE, 1 psoriatic, 1 crystal, 2 reactive and 2 seronegative oligo-arthritis). Synovial membrane cells were obtained from tissue biopsies and *ex-vivo* production of vascular endothelial growth factor (VEGF) was measured. In RA patients, 50 ml N/Saline was injected into the joint and the electrode positioned in the cavity such that the rate of oxygen consumption could be measured. Microelectrodes were also used to assess synovial pO<sub>2</sub> levels in a single metacarpophalangeal (MCP) joint in 5 RA patients. These joints were simultaneously imaged by high resolution ultrasound and power colour doppler to determine the relationship between joint architecture, vasculature and tissue pO<sub>2</sub>.

In knees, synovial tissue pO<sub>2</sub> levels were significantly lower in patients with active RA (mean = 7 mm Hg) than in patients without RA (mean = 40 mm Hg;  $P = 0.002$ ). In RA, oxygen was consumed from N/Saline introduced into the cavity at a rate of 20.5 mm Hg/min. Production of VEGF from synovial cells was greater for patients with RA (mean = 868pg/106 cells) than from synovial cells from patients without RA (mean = 84pg/106 cells;  $P < 0.01$ ).

In the 5 MCP joints studied, a total of 9 vascular areas were sampled. The mean pO<sub>2</sub> at these sites was 97 mmHg. In 19 non-vascular areas sampled, the mean pO<sub>2</sub> was 34 mm Hg (range 6-73). In a vascular erosion the tissue pO<sub>2</sub> was measured as 41 mm Hg.

In conclusion, marked hypoxia is observed in selected regions of inflamed synovium and is a likely stimulus for local VEGF production and angiogenesis. However, the increased vascularity associated with erosive damage is insufficient to restore oxygen homeostasis at the site of joint destruction.

## P29

### Cartilage-derived morphogenetic protein-1 and -2 are endogenously expressed and stimulate proteoglycan synthesis in healthy and osteoarthritic human articular chondrocytes

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**Objective:** Cartilage-derived morphogenetic protein-1 and -2 (CDMP-1 and -2) form a subgroup within the Bone morphogenetic protein family. While they are essential during embryonic joint and limb development, their role in postnatal articular cartilage is not fully clear. In this study we examined the stimulatory effects of CDMP-1 and -2 on proteoglycan synthesis and cell proliferation on postnatal human articular chondrocytes and investigated the hypothesis that osteoarthritic chondrocytes lose their responsiveness to CDMP-1 and -2 compared to healthy cells and thus lead to a decrease in proteoglycan synthesis that impairs maintenance of matrix integrity.

**Methods:** Chondrocytes were isolated from human articular cartilage from patients with and without osteoarthritic lesions. Cell number was assessed directly after collagenase digestion and chondrocytes were cultured as monolayers for a period of seven days in a chemically defined serum-free basal medium (BM) with and without the addition of recombinant CDMP-1 and -2. The proteoglycan-synthesis rate was measured by [<sup>35</sup>S]sulfate incorporation into newly synthesized macromolecules. Growth factors influence on cell proliferation was investigated by [<sup>3</sup>H]thymidin incorporation. Using RT-PCR the endogenous expression of CDMPs and their respective type I and type II receptors was examined.

**Results:** Cell number per mg tissue of osteoarthritic cartilage was significantly reduced, on an average by 45%, compared to healthy controls ( $P < 0.007$ ). CDMP-1 and -2 stimulation markedly

increased proteoglycan synthesis in postnatal human articular chondrocytes ( $P < 0.05$ ). A comparison of the biosynthetic activity between healthy and osteoarthritic samples revealed no difference, neither in stimulated nor in unstimulated cultures. [3H]thymidine incorporation showed that CDMP-1 and -2 treatment had no effect on cell proliferation. RT-PCR results indicated that CDMPs and their respective receptors are endogenously expressed not only in healthy but also in osteoarthritic cartilage.

**Conclusion:** The present study shows that CDMPs are potent stimulators of proteoglycan synthesis in postnatal articular chondrocytes and exert their anabolic effects on both healthy and osteoarthritic cartilagenous tissue. This data and the finding that CDMPs and their receptors are endogenously expressed in healthy and osteoarthritic cartilage suggest that osteoarthritis is not associated with a loss of responsiveness to CDMP-1 and -2. Moreover the decrease in cell number seems to be important for the limited tissue repair capacity in osteoarthritis.

### P30

#### Effects of cartilage-derived morphogenetic proteins and osteogenic protein-1 on osteochondrogenic differentiation of periosteum-derived cells

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Localization studies and genetic evidence have implicated Cartilage-derived morphogenetic proteins-1, -2 (CDMP-1 and CDMP-2) and osteogenic protein-1 (OP-1) in the osteochondrogenic differentiation of mesenchymal progenitor cells during embryonic development and in postnatal life. Based on their expression pattern and the evidence that periosteum contains mesenchymal cells in the cambium layer that can undergo bone and cartilage formation, we hypothesized that CDMPs and OP-1 may be involved in long bone development and fracture healing. To test this hypothesis, periosteum-derived cells from young calves were cultured as monolayers under serum-free conditions with and without the addition of recombinant CDMP-1, CDMP-2 and OP-1. Phenotypic analysis indicate that periosteum-derived cell populations prepared, expanded and cultured under the conditions described below, constitutively express mRNAs for the bone markers osteocalcin, osteopontin and collagen type I, and the chondrogenic markers collagen type II and aggrecan as determined by reverse transcription (RT)-PCR. Moreover, histologic examinations showed positive staining for alcian blue and alkaline phosphatase (AP). Treatment of periosteum-derived cells with CDMPs and OP-1 resulted in a dose-dependent increase of cell proliferation; CDMP-2 was less active in this regard. Furthermore, all growth factors enhanced osteogenic differentiation as assessed by a time- and dose-dependent stimulation of AP activity and OP-1 increased mRNA expression for osteocalcin and collagen type I. We further examined the effects of CDMPs and OP-1 on chondrogenic differentiation of periosteum-derived cells. Both CDMPs and OP-1 stimulated 35S-sulfate incorporation into newly synthesized macromolecules with OP-1 having a more pronounced stimulatory effect when compared with CDMP-1 and CDMP-2. Our results indicate that distinct members of the BMP-family act on periosteum-derived cells to increase their mitotic and metabolic activity. The enhancement of both the chondrogenic and osteogenic differentiation suggests that these growth factors might contribute to the post-natal local regulation of bone formation and fracture repair.

### P31

#### TNFA, IL10 and TGFb1 gene polymorphism in myositis and mixed connective tissue disease (MCTD)

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**Background:** The polymorphisms in the regulatory regions of cytokine genes have been considered as potential markers for disease susceptibility. Some of these polymorphisms are proved to have functional roles. These could be important for understanding the pathogenesis of myositis and MCTD, which are inflammatory diseases of unknown genetic background. Our aim was to investigate whether iV308 TNFA, -1082 IL10 and codon 25 TGFB1 gene polymorphisms associate with myositis and/or MCTD or with certain clinical and immunological parameters in these disorders.

**Patients and Methods:** 72 patients with myositis and 24 patients with MCTD were genotyped for the above markers and compared with a control group from the same population. Gene specific PCR with restriction endonuclease mapping was used for the detection of polymorphisms.

**Results:** Our preliminary data suggested that the frequency of T2 allele of TNF was significantly increased in myositis patients compared to the controls. There were 28 homozygous TNF1/TNF1 (39%), 39 heterozygous TNF1/TNF2 (54%) and 5 homozygous T2/T2 (7%) with frequency alleles 39 and 61% (TNF1 and TNF2 respectively). Regarding the MCTD patients there was a tendency for those patients who had high serum levels of TNF- $\alpha$  to carry the TNF2 allele (P value was 0.08). The frequencies of IL10 and TGFB1 alleles were not different in myositis or MCTD compared to the control group.

**Conclusion:** An increased frequency of the TNF2 allele which may be associated with high production of TNF- $\alpha$  levels was observed in myositis patients. This may be of importance for the pathogenesis of this disorders.

### P32

#### Association between TNF -308A and systemic lupus erythematosus in relation to HLA-DR3 and six microsatellite markers on the short arm of chromosome VI

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Allelic imbalance at polymorphic loci within the human HLA-DRB1 and TNF genes has been observed in association with increased susceptibility to systemic lupus erythematosus. We investigated whether the association of HLA-DRB1\*0301 (HLA-DR3) and TNF-308A with SLE could be attributed to linkage to six polymorphic microsatellites between HLA-DRB1 and HLA-C. Ninety-one consecutive Caucasian patients with SLE and 253 controls (organ donors) were typed for HLA-DRB1, D6S1014, D6S273, TNFA, MIB, C-1-2-5 and C-1-3-2 and for TNF promoter polymorphisms. Independent contribution of alleles to disease susceptibility was estimated by crosstabulation and multivariate regression.

**Results:** Carriership of TNF-308A was associated with susceptibility to SLE (odds ratio [95% confidence interval], 3.70 [2.24-6.11]). This remained present after stratification on carriership of HLA-DR3

(pooled odds ratio, 2.53 [1.37-4.70]). Stratification further revealed a possible association of carriage of C-1-2-5\*192 with protection from SLE beyond the effects of HLA-DR3 and TNF-308A. Gene dose effect was observed for -308A only (homozygotes, 7.75[3.01-20.0], heterozygotes, 3.15[1.85-5.37]). In multivariate analysis, the association between HLA-DR3, TNF-308A, and C-1-2-5\*192 remained independently associated with susceptibility to SLE (2.58 [1.29-5.18], 2.76 [1.43-5.31], and 0.26[0.10-0.66], respectively).

**Conclusion:** An association of carriage of TNF-308A with susceptibility to SLE can not be attributed to linkage to HLA-DR3, nor to other polymorphic markers in the vicinity of the TNF gene. Further loci that are independently associated with SLE might be located in the vicinity of marker C-1-2-5.

**P33**

**A single nucleotide polymorphism on the IL-10 locus defines an expression polymorphism and a possible risk factor to develop RA**

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**Introduction:** IL-10 production differs between individuals. We have evaluated the IL-10 production in whole blood cultures with/without LPS. The comparison of monozygotic twins, sibs and unrelated individuals yielded an estimate of heritability of 70% (Lancet 98). Moreover the ex-vivo IL-10 production was associated with haplotypes defined by alleles of CA-repeats (PNAS 98). In line with these results we have demonstrated that the interindividual differences in production of mRNA encoding IL-10 are similar than the interindividual differences in IL-10 protein production (Rheumatology 2000).

**Aim:** A) to define SNPs associated with common haplotypes. B) to study the association of IL-10 production with haplotypes/SNPs. C) to measure the distribution of IL-10 SNPs in RA versus controls. **Methods:** DNA of high and low IL10 producers were sequenced. Subsequently, the association between LPS-induced IL-10 production and previously described (Lancet 98) panel was analyzed.

**Results:** The following SNPs were identified: -3575 A to G, -2849 A to G, -2763 A to C and -1330 A to G. Previously (Genes and Immunity 99) we have identified 4 ancestral IL-10 haplotypes. The current SNP's on: IL10.1 R3-AAAA-(IL10G)-GCC, IL10.2 R2-TGCG-(IL10G)-ACC, IL10.3 R2-AGAA-(IL10G)-GCC, and IL10.4 R2-TGCC-(IL10G)-ATA. To investigate whether these SNP's were functional we analyzed the LPS-induced IL-10 production of 161 healthy donors with a specific genotype: -3575: AA (n = 38) 1896 ng/ml, AT (n = 76) 3232 ng/ml, TT (n = 47) 3195 ng/ml. -2849: AA (n = 21) 2115 ng/ml, AG (n = 75) 2950 ng/ml, GG (n = 65) 3111 ng/ml (Mann-Whitney test both P < 0.05). Next, the analysis was repeated in a different group of donors: 135 partners of patients with SLE/MS: -3575: AA (n = 29) 4190 ng/ml, AT (n = 71) 4521 ng/ml, TT (n = 35) 4401 ng/ml (MW-test P = 0.6).

-2849: AA (n = 26) 3845 ng/ml, AG (n = 41) 4577 ng/ml, GG (n = 68) 4543 ng/ml (MW-test P = 0.04, for G carrier versus non G: P = 0.02). Next, the distribution of -2849 SNP was compared in RA patients compared to controls. Control-Panels were 1) partners of MS-SLE patients (n = 135) and 2) organ donors (n = 168). RA-patients were: 1) incident RA cases, 2) outpatient consecutive RA and 3) RA patients from our early arthritis cohort.

**Conclusion:** We (Eskdale et al, Lancet 98) have previously found that the allele IL-10R3 microsatellite was less frequent in three ethnic groups of RA patients (Afro-americans P < 0.01, English P < 0.008 and Scottish P < 0.02). The SNP that defines the haplotype on which R3 is located is also less prevalent in three groups of

|    | Control |     | RA Patients |     |     |
|----|---------|-----|-------------|-----|-----|
|    | (1)     | (2) | (1)         | (2) | (3) |
| AA | 27      | 16  | 3           | 24  | 16  |
| AG | 42      | 64  | 38          | 141 | 74  |
| GG | 71      | 88  | 51          | 152 | 91  |

Total chi-square: P = 0.0014

dutch RA compared to two groups of dutch controls. These data suggest that a high innate IL-10 production is a risk factor for RA. This may be due to the B-cell stimulating properties of IL-10.

**P34**

**Elevated levels of soluble intercellular adhesion molecule -1 in systemic lupus erythematosus**

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**Objective:** To assess the value of measuring serum levels of soluble intercellular adhesion molecule 1 (sICAM-1) in systemic lupus erythematosus (SLE).

**Material and methods:** We studied 35 patients (pts) (7 female, 28 male), satisfying the ACR criteria for SLE. Mean age of pts was 31,4±12,0 years (range 17-63), mean disease duration was 81,8±70,5 month (range 2-240). Disease activity was assessed by disease activity indices (SLAM, SLEDAI). Enzyme-linked immunosorbent assay was used to measure levels of sICAM-1 (R&D, USA). The results were compared with 18 healthy subjects.

**Results:** Levels of sICAM-1 were found elevated (more than 2 SD above the mean in normal controls, 443 ng/ml) in 7 of 35 (20%) pts with SLE. The relations between positive sICAM-1 and some clinical manifestations of SLE have been detected. We found significant correlation between individual sICAM-1 serum level and the SLEDAI (r=0.43) and SLAM (r=0.56) scores, and ESR (r=0.53).

| Parameters, (%) or mean ±SD | Positive sICAM-1 (n = 7) | Negative sICAM-1 (n = 28) | P      |
|-----------------------------|--------------------------|---------------------------|--------|
| ICAM-1, ng/ml               | 512,3±45,5               | 284,3±85,0                | <0,001 |
| Malar rash                  | 28,6%                    | 14,2%                     | NS     |
| arthritis                   | 42,8%                    | 25%                       | NS     |
| nephritis                   | 57,2%                    | 32,1%                     | NS     |
| CNS involvement             | 71,4%                    | 39,3%                     | NS     |
| Serositis                   | 42,8%                    | 14,3%                     | NS     |
| ESR, mm/h                   | 38,0±24,8                | 21,2±16,1                 | <0,05  |
| SLAM, score                 | 16,0±7,4                 | 9,03±5,7                  | <0,05  |
| SLEDAI, score               | 18,0±12,4                | 10,2±7,75                 | <0,05  |

**Conclusion:** Elevated serum levels of sICAM-1 can be found in SLE and correlate with disease activity. Longitudinal studies may establish their clinical value in the monitoring or the prognosis of patients.

## Poster Discussion C

### Gene Regulation and Genetics

#### P35

#### Spontaneous activation of JNK-1 and PI-3 kinase can be induced in lupus-like chronic GVHD in the P->F1 model

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Abnormalities in intracellular signaling pathways have been described in human SLE however, it is not clear whether they are primary, predisposing events or secondary to SLE expression. To address this question, we used a murine model of SLE (chronic P->F1 GVHD) and compared the function of a broad range of intracellular signaling pathways. In this model, SLE-like disease is induced in normal F1 mice by injection of parental CD4+ T cells and is mediated predominantly by Th2 cytokines. In contrast, acute GVHD is a cell-mediated anti-host response induced by CD4 and CD8 donor T cells and both Th1 and Th2 cytokines. Spleen cell lysates from control and GVHD mice (day 10 and 21) were immunoprecipitated with antibodies to Raf-1, ERK-1, JNK-1, p38 MAPK and PI-3 kinase and the function of the precipitated kinases determined using a specific substrate for each kinase. Raf-1 and ERK1 were selectively increased in acute GVHD (2-fold) only, whereas JNK-1 and PI-3 kinases were increased in both acute and chronic GVHD. This increase was approximately two fold greater for acute vs. chronic GVHD mice for both kinases. p38 MAPK was not increased over control in either form of GVHD and may reflect the earlier peak of IL-2 (day 3) in this model. The data suggest that Raf-1 and ERK-1 may be important in CD8 driven cell mediated immune responses whereas JNK-1 and PI-3 kinase signaling may be important in CD4 driven antibody mediated immune response. Studies are under way using purified T cell subsets to confirm this hypothesis. However these data support the idea that the signaling abnormalities reported in human SLE may be secondary to a continuous immune activation rather than indicative of a primary predisposing event.

#### P36

#### T lymphocytes of patients with SLE lack Stat4

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**Background:** Signal transducers and activators of transcription (Stats) are essential for cytokine receptor signaling. Stat4 is found in lymphocytes and activated monocytes, where it is tyrosine-phosphorylated after IL-12 or (human) interferon-alpha bind their respective receptors. Stat4 plays a significant role in the development of Th1 cells: Stat4<sup>-/-</sup> mice lack such cells and are Th2-shifted. Moreover, Stat4 expression is known to be influenced by lymphocyte activation. We therefore investigated the expression of Stat4 in human SLE.

**Methods:** Peripheral venous blood was drawn from 9 patients fulfilling ACR criteria for SLE and 7 healthy controls. Protein lysates were prepared from highly enriched T cells and standardized for protein, electrophoresed on polyacrylamide gels and electrotrans-

ferred. Stat4 was detected using polyclonal antibodies, peroxidase-conjugated secondary antibodies and chemoluminescence.

**Results:** Stat4 protein was clearly detectable in 5 out of 7 healthy controls. In contrast, the T cells of only 1 of 9 SLE patients contained Stat4. This difference was statistically significant ( $P = 0.035$  in Fisher's exact test). This result was independent of disease activity or therapy. Lysates from crude PBMC lysates gave similar results, which would be expected, given the normal cellular distribution of Stat4, but some SLE lysates contained additional bands of similar size.

**Conclusion:** The lack of Stat4 protein in SLE T lymphocytes may have immunological consequences by hampering Th1 answers. It is unclear at the moment whether the differences observed are due to constant immune stimulation.

#### P37

#### Global analysis of gene expression in unseparated and CD8+ cells from bronchoalveolar lavage of patients with scleroderma lung disease

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The molecular mechanisms that lead to lung fibrosis following lung inflammation in patients with scleroderma are unknown. The objective of this study was to identify patterns of abnormal gene expression in unfractionated bronchoalveolar lavage (BAL) cells from scleroderma patients. DNA microarrays were used to assess expression of over 4000 genes in BAL cells from 17 scleroderma patients and 7 controls. Hierarchical matrices were constructed and showed that BAL cell samples from patients with lung inflammation segregated into one cluster, whereas BAL cell samples from patients without lung inflammation and controls clustered in another. Next, 372 genes were identified that were expressed at least two-fold higher or lower in BAL samples from patients without lung inflammation, compared to controls. These genes, which may represent a scleroderma phenotype independent of tissue inflammation, clustered into eight groups. One cluster of note included receptors for several chemokines, IL-1, IL-13, IL-18, and IFN-gamma receptors, as well as IL-10, IL-12, macrophage stimulating factor, VEGF, IGF binding protein and TXK tyrosine kinase. Fibroblast growth factors, other cytokines, and intracellular signaling molecules were among the genes in other clusters. Finally, 238 genes were identified that were over- or under- expressed in BAL cells from patients with greater risk of lung fibrosis, that is, patients with lung inflammation, compared to both patients without lung inflammation and controls. These genes clustered into 3 groups which included genes induced by stress (such as heme oxygenase, complement components, and heat shock proteins), multiple chemokines (such as MCP-1, MIP-1, and PARC), and genes associated with several intracellular signaling pathways (such as JNK2 kinase, diacylglycerol kinase, and phosphatidylinositol 4,5 bisphosphate 5-phosphatase). Similar studies of global gene expression have begun with CD8+ T cells isolated from BAL samples. Preliminary data suggest abnormal expression of lymphotoxin beta, endothelin-2, fibroblast growth factors, and TGF-beta RII. In summary, scleroderma patients with lung inflammation have distinct patterns of gene expression in BAL cells, compared to patients without lung inflammation and healthy controls. Cluster analyses of genes that are abnormally expressed in different patient groups may shed light on new mechanisms that contribute to the development of the scleroderma phenotype, including genes likely to be involved in the inflammatory process and subsequent development of fibrosis in this autoimmune illness.

### P38

#### Redox-sensitive changes in conformation and cellular localization of LAT and downstream TCR signaling lead to hyporesponsiveness of synovial fluid T cells in rheumatoid arthritis

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In rheumatoid arthritis (RA), the synovial fluid (SF) T lymphocytes present in the inflamed joints, display hyporesponsiveness upon engagement of the TCR/CD3 complex despite phenotypic evidence of former activation. We have previously shown that the central and crucial adaptor protein LAT (linker for activation of T cells), which plays a central and crucial role in the T cell receptor (TCR)-mediated signaling pathways, exhibits deficient phosphorylation due to displacement of the integral membrane protein from the plasma membrane in SF T lymphocytes. SF T lymphocytes exhibit several features of chronic oxidative stress, e.g. severely decreased intracellular levels of glutathione (GSH), and our previous studies have indicated that the subcellular localization of LAT is sensitive to changes in the intracellular GSH levels. The cysteine-to-serine substitutions of several cysteine residues (C26/29 or C117) within LAT creates LAT mutants that are resistant to reduced intracellular GSH levels and remain membrane-anchored in GSH-depleted cells.

In this study, we have used the redox-insensitive LAT mutants to study the effect of redox balance alterations, like in SF T lymphocytes, on TCR signaling pathways downstream from LAT and on CD28 signaling pathways. In co-transfection experiments, we show that the presence of the redox-insensitive LAT mutants allows for the partial restoration of the TCR-mediated signaling pathways, but not the signaling pathways induced through the CD28 receptor. The data are indicative that the Raf1-ERK and the calcium-calmodulin pathways leading to transcriptional activation of AP-1 and NFAT, respectively, are very sensitive to reduced intracellular GSH levels, while the activation of the p38/Mpk2 pathway leading to AP-1-mediated transcription is mostly unaffected by chronic oxidative stress. A very proximal event in the CD28-mediated signaling pathways seems to be extremely sensitive to GSH depletion since costimulation did not affect the transcriptional activity of either AP-1 or NF- $\kappa$ B.

We conclude that the signaling pathways in SF T lymphocytes from RA patients are affected at several levels by chronic oxidative stress, all contributing to the observed hyporesponsiveness of these cells.

### P39

#### Peripheral corticotropin releasing hormone signaling is mediated by Type 1 $\alpha$ receptors in early human inflammatory arthritis

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Corticotropin Releasing Hormone (CRH) is essential for modulating the effects of the inflammatory response *in vivo*. Elevated levels of CRH are produced locally in inflamed human synovial tissue and observations indicate a role for CRH in the pathogenesis of inflammatory joint disease. CRH action is initiated by two distinct subtypes of CRH receptors, CRH-R1 and CRH-R2, which are approximately 68% homologous. Each subtype exhibit spliced vari-

ants ( $\alpha$  and  $\beta$ ), displaying pharmacologically and functionally distinct isoforms.

To further elucidate the peripheral biological role for CRH we examined the expression of known CRH receptor subtypes in inflamed human synovium ( $n = 14$ ) and compared the expression patterns to normal synovium. Immunohistochemistry and RT-PCR confirmed enhanced expression of CRH-R1 receptors in rheumatoid (RA) and psoriatic (PsA) arthritis synovial tissue. In all tissues studied CRH R1 $\alpha$  mRNA was identified, however, we were unable to detect other CRH R1 or CRH R2 isoforms in the same cohort of patients. Immunoreactive CRH-R1 is abundantly expressed on vascular endothelial cells and discrete perivascular cell populations, positively identified as mast cells. In contrast, in normal synovial tissue, neither CRH receptor subtype is expressed.

Selective up-regulation of CRH receptors in inflamed synovial tissue indicates that CRH functions locally, in an autocrine/paracrine receptor-mediated response. Our findings suggest that CRH signaling, via CRH-R1 $\alpha$ , may play a role in both vascular changes and pathologic mechanisms associated with joint inflammation.

### P40

#### Detection of the "Kreiser" (maf B) gene by combination of in situ-hybridization and immunohistochemistry of RA-, osteoarthritis- and normal controls-synovial tissue samplings as a potential significant marker for early RA

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**Introduction:** By analyzing gene expression profiles of arthritic tissue on DNA microarrays (EOS) compared to the "Body Atlas", a reference database of 13 normal human tissues, we found the RAB3 "Kreiser" (maf B) gene (member of the maf gene family and encoding for a transcription activator specific for mesenchymal and neuronal organogenesis) highly expressed in early rheumatoid arthritis (RA) (< 2 years disease duration).

**Objective:** To investigate the functional "Kreiser" gene expression in RA-, osteoarthritis- and normal controls- synovial samplings by combination of in situ-hybridization and immunohistochemistry.

**Methods:** We analyzed synovial biopsies ( $n = 12$ ; 7f/5m) from 5 RA- (3 early RA, 2 RA), 4 osteoarthritis-patients and 3 normal controls, which were taken by arthrotomy by various indications and miniarthroscopy of MCPs (2 early RA). Samples were analyzed by in situ-hybridization with the "Kreiser" (maf B) gene-mRNA and immunohistochemistry (e.g. Ki 67, CD 68).

**Results:** We detected increased "Kreiser"-mRNA levels in 3 early RA samples in the synovial lining layer and no signals in the control and compared samples. At higher concentrations (>1ng/ $\mu$ l) of RNA-oligonucleotides unspecific hybridization-signals prevailed in tissues of all diseases (even in normal controls). The combination of both methods (in situ-hybridization and immunohistochemistry) identifies the single cells inside the synovial lining layer which contains the highly expressed RAB3 "Kreiser" (maf B) gene.

**Conclusion:** Based on the gene expression profiles through oligonucleotid-microchip-array-analysis by EOS and the detection of the increased "Kreiser" (maf B) gene expression in combination of in situ-hybridization and immunohistochemistry of RA-synovial tissue samplings we discuss the "Kreiser"-gene as a potential inducing element in the pathogenesis of early RA. Further serial studies are needed to clarify the significance of "Kreiser" especially for early RA and the molecular pathogenesis of this disease.

**P41****Defective Rap1 activation in synovial fluid T cells from patients with rheumatoid arthritis.**

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**Background:** Rap1 is a small G-protein, member of the Ras GTPase family. Rap1 has been linked to T cell anergy and it is suggested that Rap1 has the potential to inhibit Ras-mediated oncogenic or growth promoting activity. Synovial fluid (SF) T cells from patients with rheumatoid arthritis (RA) display a defective phosphorylation pattern of several pivotal signaling proteins and severe hyporesponsiveness upon antigenic stimulation. Our previous data showed that the hyporesponsive state of the SF T cells in RA correlates with markers of oxidative stress and replenishment of the intracellular level of the anti-oxidant glutathione (GSH) by treatment with N-acetyl-L-cysteine (NAC) restores the observed signaling defects.

**Objective:** To determine Rap1 activation and its correlation with oxidative stress in T cells from healthy controls compared to peripheral blood (PB) and SF T cells isolated from RA patients.

**Methods:** T cells from healthy donors and PB and SF T cells from 5 RA patients were isolated and after 5 minutes of stimulation with either anti-CD3 antibodies or PMA+ionomycin, whole cell lysates were prepared in Ral lysis buffer. GTP-bound Rap1 was isolated using the bacterial expressed fusion protein GST-RalGDS and detected by ECL Western blotting. In whole cell lysates, total Rap1, rapGAP and Spa1 were detected by ECL western blotting as well.

**Results:** Our data show that in T cells isolated from healthy controls, Rap1 was activated in a redox-dependent way. Upregulation of intracellular GSH levels by incubation with 10 mM NAC for 48 h resulted in diminished Rap1 activation, while depletion of GSH by pre-incubation for 48h with 200  $\mu$ M BSO resulted in enhanced Rap1 activation. We also observed that treatment of T cells with H<sub>2</sub>O<sub>2</sub> led to the rapid activation of Rap1.

Despite an environment of oxidative stress in the inflamed joints of RA patients, we found that in SF T cells Rap1 was present in its inactive GDP-bound state. Furthermore, upon stimulation Rap1 remained inactive in SF T cells while in contrast Rap1 could be activated in PB T cells from RA patients. The defective Rap1 activation was not due to increased levels of the Rap1 GTPase activating proteins RapGAP or Spa1. While restoration of the intracellular redox balance does reverse the hyporesponsiveness of the SF T cells, the replenishment of GSH with NAC had no effect on the defective Rap1 activation.

**Conclusions:** 1. In healthy T cells the small GTPase Rap1 is activated by H<sub>2</sub>O<sub>2</sub>; 2. Rap1 activation after T cell stimulation is redox-dependent; 3. Rap1 activation is defective in SF T cells from RA patients, and cannot be restored by the replenishment of GSH with NAC.

**P42****The RIIbeta-subunit of protein kinase A (PKA) inhibits c-fos synthesis in T cells**

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In human primary T cells, the type II isozyme of protein kinase A (PKA-II) is localized to cytoskeletal elements or organelle membranes. Stimulation of T cells via the T cell receptor/CD3 complex

or by addition of the cAMP analog, 8-Cl-cAMP, activates PKA-II, resulting in nuclear translocation of the RIIbeta-subunit from the cytosol and apparent RIIbeta DNA-binding. In current experiments, we demonstrated that recombinant RIIbeta forms a heterodimer with recombinant CREB, a nuclear transcription factor, as shown both by EMSA and immunoprecipitation/immunoblotting. We found no evidence of direct binding of RIIb to c-fos-defined CRE oligonucleotides by EMSA. Although the RIIbeta-CREB heterodimer binds to the c-fos cAMP response element (CRE), phosphorylation of both RIIbeta and CREB by PKA enhances binding to c-fos CRE oligos. *In vivo*, phorbol myristate acetate (PMA)-induced synthesis of c-Fos protein is inhibited by DNA-binding of RIIbeta-CREB complexes. Taken together, these data suggest that, in addition to its primary function as an inhibitor of catalytic-subunit activity, the RIIbeta-subunit also acts as a transcription factor that can modulate the activity of the c-fos promoter. Therefore, we propose that RIIbeta may be a transcriptional repressor of c-fos.

**P43****Invasion of synoviocytes is inhibited by gene transfer of TNF-BP or IL10 in an *in vitro* invasion model**

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In RA fibroblast like synoviocytes (FLS) degrade and invade into adjacent cartilage. An *in vivo* model is the SCID-mouse/ Human cartilage /synoviocyt model (S. Gay). Previous studies indicate that the invasive behaviour of fibroblast like synoviocytes can be tested *in vitro* in a matrigel transwell system. Matrigel, mainly composed of laminin and collagen IV, serves as a model for cartilage.

The aim of this study was to compare the invasive behaviour of FLS from RA and osteoarthritis (OA) patients and to investigate the effect of adenoviral (Ad) transfer of genes encoding IL-10 and TNF-binding protein (p55) (TNF-BP) on the invasive behaviour of FLS from RA patients.

FLS from 43 RA and 28 OA patients obtained from synovial tissue harvested at joint replacement surgery, were seeded at confluency in serum free medium on top of a matrigel coated transwell filter with 8  $\mu$ m pores. Medium with 10 % Fetal Calf Serum and 10 % Human Serum was used in the lower compartment. Three days post incubation cells were fixated, stained and the invaded synoviocytes on the lower side of the filter were counted. To test the effect of IL10 and TNF-BP, RA synoviocytes were infected overnight with 5, 10, 50 and 100 plaque forming units (pfu)/cell of Ad.IL10, Ad.TNF-BP or Ad.luciferase (negative control) and then tested in the invasion model. Significantly more RA synoviocytes invaded through the matrigel (median = 4035 cells) as compared to OA synoviocytes (median = 1900 cells;  $P < 0.001$ ). IL10 and TNF-BP gene transfer both resulted in a dose dependent inhibition of invasion with a maximal inhibition of 93.7% ( $\pm 10.7$ ) and 86.6% ( $\pm 14.5$ ) respectively, while luciferase gene transfer showed a maximal inhibition of 17.6% ( $\pm 1.7$ ).

In conclusion, the invasive behaviour of FLS can be studied in the matrigel transwell system. This assay discriminates between the invasive behaviour of RA and OA FLS. The invasive behaviour of RA synoviocytes can be strongly inhibited by IL10 indicating that IL10 is able to downregulate the proteins involved in invasive growth. The inhibitive effect of TNF-BP indicates that continuous production of TNF is involved in invasive behaviour of synoviocytes.

## P44

### Specific suppression of the transcription factor AP-1 by mepacrine

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Mepacrine has been used for decades and the beneficial effects of this drug are well described. Since endothelial cells (EC) are in many cases the first cells to come in contact with drugs, the effect of mepacrine on certain aspects of EC biology were studied. First, our data demonstrate that at high doses mepacrine can have a marked impact on the integrity of the EC monolayer without grossly interfering with cell viability. The described impact of mepacrine on EC might explain, at least in part, the negative effects of this drug observed in the past. More importantly, mepacrine profoundly effects gene regulation in EC and fibroblasts. Mepacrine binds to DNA in a sequence specific manner. While NF- $\kappa$ B-DNA interactions are not effected, AP-1-DNA binding is blocked by mepacrine. Such differential effects are presumably due to sequence specific intercalation of mepacrine into the AP-1 consensus element. Pre-incubation of oligonucleotides resembling this sequence blocked the subsequent binding of nuclear extract containing AP-1 protein(s). Consequently, mepacrine prevents the upregulation of genes which depend mainly on the activation of AP-1. One of the few genes which have been found to depend heavily on the activation of this transcription factor is metalloproteinase-1 (MMP-1). We demonstrated by western blot that treatment of fibroblasts with mepacrine completely prevented subsequent upregulation of MMP-1. Since MMP-1 plays an important role in the propagation of rheumatic diseases, we suggest that the beneficial effect of mepacrine seen in the past is due, at least in part, to the described mechanisms.

## P45

### Patterns of differentially expressed genes in synovial tissue from RA and OA patients and from normal joints

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**Objective:** To identify key genes in the pathomechanism of rheumatoid arthritis (RA), synovial tissues from RA, osteoarthritis (OA) and from normal joints (ND) were compared by a subtractive hybridization technique, the representational difference analysis (RDA).

**Methods:** Synovial tissues from 3 RA, 3 OA patients and 5 normal joints were selected according to their disease-characteristic immunohistochemical findings and to their expression of high versus low levels of inflammatory (IL-1 $\beta$ , TNF- $\alpha$ ) and destructive markers (MMP-1, MMP-3) as determined by semiquantitative RT-PCR. Pooled mRNA from RA, OA and normal tissues was transcribed, digested by a 4-base-cutter, ligated to adapter-primers and amplified to form representational amplicons. Subtractive hybridizations were performed by different protocols: 1. the OA amplicon (driver) was subtracted from the RA representation (tester); 2. the RA (driver) from the ND (tester) and 3. the ND (driver) from the OA representation (tester). Using primers specific for the corresponding tester, the difference-products were selectively amplified, cloned, sequenced and compared to published sequences in the Genebank. Differential expression of identified genes was validated by semiquantitative RT-PCR.

**Results:** Approximately 150 genes were found to be differentially expressed in RA synovial tissue as compared to OA or ND tissues respectively, or in OA tissues as compared to ND. Interestingly, some genes were identified to be overexpressed in both groups: RA (i.e. difference-product from RA minus OA) and OA (OA minus ND), indicating rather an association to general joint destruction than to RA-specific mechanism. Other genes were found to be differentially expressed only in the RA representation. 30 of the differentially expressed genes identified from each disease group were analyzed in synovial tissues from further 20 RA, 20 OA patient and 20 normal joints. The expression of some genes showed either a significant correlation to those of inflammatory genes (IL-1 $\beta$  and TNF- $\alpha$ ) or to those of destructive markers (MMP-3).

**Conclusions:** The analysis of differential gene expression in chronic joint diseases is a promising approach to identify deregulation of the inflammatory network to explain the inappropriate immune response with autoaggressive outcome. Furthermore a pattern of genes is generated which is specifically or preferentially expressed in RA. Such patterns will be of diagnostic value, especially for disease characterization, longitudinal studies and analysis of therapeutic effects.

## P46

### MMP-1, MMP-3 and MMP-10 are involved in the degradation of cartilage

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Rheumatoid arthritis (RA) is characterised by degradation of cartilage and invasion of fibroblast-like synoviocytes (FLS) into adjacent cartilage. Several families of proteinases are involved in the degradation of cartilage, especially the matrix metalloproteinases (MMP's) and cathepsin K. However, it is not known which MMP's are responsible for the degradation of cartilage and the invasiveness of FLS. In this study, the expression of MMP's 1 to 20 and cathepsin K in cultured FLS obtained from joint replacement surgery from RA, osteo-arthritis (OA) and other non-destructive arthropathies are investigated and compared to the invasiveness of the FLS in a matrigel transwell system. In this matrigel transwell system previous studies have shown that FLS from RA patients were significantly more invasive than FLS from patients with OA or other non-destructive arthropathies.

FLS from synovial tissue of 32 RA, 18 OA and 14 patients with other non-destructive arthropathies were obtained from joint replacement surgery. The FLS were grown to confluency and RNA was isolated at passage 1 or 2. cDNA was synthesized using oligo-dT and reverse transcriptase. Expression of MMP's and cathepsin K was investigated using RT-PCR. For MMP's 2, 3, 7-12, 14-17, 19, 20 and cathepsin K RT-PCR was performed with primers for the MMP under investigation and primers for beta-actin in one mix. For MMP-1 and 13 no primers for beta-actin were in the mix. The intensity of the bands were compared and given a number from 0 (no expression) to 3 (intensity more than beta-actin). These numbers were related to the invasiveness (number of cells) in a matrigel transwell system.

FLS that expressed MMP-1, MMP-3 or MMP-10 were significantly more invasive (median number of invasive cells: 3970, 4525, 4998, respectively) than cells that did not express MMP-1, MMP-3 or MMP-10 (1826,  $P = 0.02$ ; 3081,  $P = 0.01$ ; 2537,  $P = 0.01$ , respectively). Expression of the other MMP's and cathepsin K did not show a significant relationship with invasive growth. Expression



of MMP-9 showed a trend with higher expression in more invasive cells ( $P = 0.09$ ). From this study it can be concluded that a correlation exists between expression of MMP-1, MMP-3 and MMP-10 and invasiveness of FLS in a matrigel transwell system.

#### P47

### The release of an ERK-activating factor from cartilage explants in response to trauma

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Mechanical injury to cartilage predisposes to premature degenerative arthritis but little is understood of the chondrocytic response to injury at the cellular and molecular level. We have shown that the extracellular regulated kinase (ERK), the original mitogen activated protein (MAP) kinase, is strongly activated in porcine articular cartilage upon scarification *in vivo*, or following cutting of rested cartilage explants *in vitro*. Activation occurs within minutes and is sustained for 24-48h. It appears to be mediated by a soluble factor which is released into the culture medium by damaged cartilage. The factor is thermolabile and retained by dialysis membrane (10kDa cut-off). We have purified the factor through a series of chromatography steps (anion and cation exchange, gel filtration and agarose-heparin columns), and are awaiting identification. It represents a potential homeostatic mechanism in response to injury, and could play a role in normal metabolism and degenerative arthritis.

## Poster Discussion D

### Cellular Immunity

#### P48

### In vitro autoreactivity against autologous keratinocytes in patients with rheumatoid and juvenile idiopathic arthritis

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In patients with rheumatoid arthritis or juvenile idiopathic arthritis we observed the tendency of peripheral blood mononuclear cells to induce graft versus host disease like histopathological changes of grade II or above (evaluated according to standard Lerner's classification) when co-cultured *in vitro* with autologous skin explants. The aim of this study was to verify if observed skin damage was really of an autoimmune origin and we also tried to compare results with autoreactivity directed against autologous synovium. We suppose that humoral as well as cellular autodestructive mechanisms may be involved in the pathogenesis of observed skin damage.

**Methods:** To prove this hypothesis (cellular autodestruction) we used  $^{51}\text{Cr}$  release cytotoxic test where peripheral blood mononuclear cells were co-cultured with autologous synovial cells as well as with autologous keratinocytes.

**Results:** We found that patient's peripheral blood mononuclear cells lysed both autologous keratinocytes (specific lysis 60%) as well as autologous synovial cells (59% specific lysis). No specific lysis of autologous keratinocytes and synovial cells was observed in healthy controls.

We suppose that peripheral blood mononuclear cells might recognise similar autoantigen(s) expressed on epidermal cells that might give rise an autoimmune response in synovium.

#### P49

### Enrichment of CD8+ CD28- cytotoxic T cells in circulating lymphocytes of patients with ankylosing spondylitis

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**Introduction:** In patients with ankylosing spondylitis (AS), HLA-B27 restricted cytotoxic T lymphocytes (CTLs) exist with specificity for arthritogenic bacteria, viral peptides or autoantigens. These MHC-class I restricted CTLs could maintain the inflammatory process even after the bacterial pathogen itself had been eradicated by antibacterial immune responses and thus be directly involved in the pathogenesis of spondylarthropathies. Phenotypically they are characterized by CD28-negativity and CD57/CD11a high-positivity. This study was performed to directly compare the relative number of CD8+ CTLs from AS patients with age-matched healthy controls. Until now only few data are available for the incidence of CD8+CD28- T cells in autoimmune diseases.

**Methods:** AS patients were recruited and examined at the Gasteiner Heilstollen Hospital. Controls were preselected by evaluating the proband's history and physical examination excluding an inflammatory or autoimmune diseases. Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation, triple-stained for CD3, CD4, and CD28-antigens and analyzed on a FACScan flow cytometer.

**Results:** Peripheral blood was analyzed from 95 AS patients and 53 age-matched healthy controls (49,1 +/- 11,4 and 48,0 +/- 14,0 years old, respectively). In AS patients CD8+CD28- T cells are expanded more than in the age-matched population (41,2 +/- 17,7% and 18,6 +/- 7,6% ( $P < 0,0001$ ), with regression lines  $y = 0,23x + 29,85$  ( $R^2 = 0,02$ ) and  $y = 0,03x + 17,03$  ( $R^2 = 0,004$ ), respectively). The percentage of CD8+CD28- T cells does not increase over the decades in healthy controls. Trends are described for increased percentages of CD8+CD28- T cells in patients with severe disease.

**Conclusions:** These data suggest that the fraction of CD8+CD28- T cells is not only increased in certain infectious diseases but also in patients diagnosed with AS. Increased percentages of CD8+CD28- T cells during ageing might be an artificial effect of undiagnosed infectious or autoimmune diseases. This finding further supports the hypothesis that increased levels of CD8+CD28- T cells can be considered pathogenic, comparable to benign monoclonal gammopathy.

#### P50

### Differences in B cell regulation in DRB1 shared epitope positive and negative rheumatoid arthritis

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**Introduction:** Aim of the study was the analysis of systemic B cell activity and of the size of the B lymphocyte compartment in patients with rheumatoid arthritis (RA)

**Material and methods:** In 94 patients with RA according to the 1987 ACR criteria, clinical, radiographic and laboratory data were gathered in a cross-sectional, retrospective study. Besides standard laboratory test, concentration of serum IgM and IgG were determined. In peripheral blood mononuclear cells, the percentages of CD4+, CD8+ and CD19+ lymphocytes were determined by dual-color flow cytometry. For all patients, the presence of the RA associated shared epitope (SE) was determined by HLA DRB1 genotyping.

**Results:** The analysis of CD19+ B cell frequencies of RA patients revealed a bimodal distribution in the study population separating one group of patients with B cell counts below 8.5 % of all lymphocytes (B cell low patients, 62 % of the study population) from a second group with more than 8.5 % B cells (B cell high, 38 %). HLA genotyping revealed, that the two groups were immunogenetically distinct. B cell low patients were more frequently SE positive than B cell high patients (84.5 % vs. 50 %,  $P < 0.001$ ), and SE positive patients had lower CD19 percentages in the rank-sum analysis when compared to SE negative ones (6.3 % vs. 14.0 %,  $P < 0.001$ ). Comparative analysis of a healthy control group showed, that B cell frequencies were diminished in SE positive and increased in SE negative patients.

B cell low patients were found to have significantly lower concentrations of RF IgM, RF IgA, and serum IgM, but not of serum IgG, when compared to the B cell high group. Multivariate analysis revealed the presence of low B cell counts to be associated with the presence of the shared epitope sequence, RF IgM seronegativity and low concentrations of serum IgM, but not with disease activity, gender, age at disease onset or disease duration.

**Conclusion:** We have found a diminished size of the peripheral B cell pool in SE positive RA patients, that is associated with lower RF IgM titers and a suppression of the parameters of polyclonal IgM, but not IgG secretion. Suppression of polyclonal autoreactivity in SE positive RA patients by clonal deletion of autoreactive, IgM+ B lymphocytes is one possible explanation for decreased B cell counts in RA.

## P51

### In adjuvant-induced arthritis the disease-triggering adjuvant squalene accumulates in draining lymph nodes but not affected joints

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Nonspecific stimulation of the immune system by adjuvants can cause joint-specific inflammation in rats, as exemplified by arthritis induced with the endogenous cholesterol precursor squalene (C<sub>30</sub>H<sub>50</sub>). To determine the uptake and distribution of injected adjuvant, and more specifically to determine whether adjuvant accumulates in affected peripheral joints, tritium-labelled squalene was used to induce arthritis in arthritis-prone DA rats. All organs, including hind paws and the site of injection, were collected at different stages of disease development. The deposition of oil was subsequently quantified by dissolving the tissues followed by scintillation counting.

The majority of injected oil never leaves the injection site, and no adjuvant oil is accumulated in the peripheral joints. Organ samples taken early prior to clinical disease and after arthritis onset displayed a similar distribution of oil, except for the draining lymph nodes and the intestines. In the draining lymph nodes, the deposition of oil accumulated over time, whereas the reverse was the case for the intestines.

A passive transfer of squalene-induced arthritis with lymph node cells was successfully accomplished, both with cells from draining inguinal lymph nodes and cells from lymph nodes not draining the injection site (axillary). Since uptake of squalene was minimal in axillary lymph nodes, this result indicates that the oil need not be present for passive transfer of the disease.

In conclusion, we report an accumulation of the arthritis triggering squalene in the draining lymph nodes but not in the peripheral joints

from the time of injection to the disease onset. This uptake evokes a systemic immune activation of unknown mechanisms that subsequently lead to a joint specific inflammation.

## P52

### p205 induces the production of rheumatoid factors

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The p205 autoantigen is the strongest stimulatory antigen for T cells known in rheumatoid arthritis (RA). It contains an 11 aminoacid stretch identical to a sequence (278-288) located in the CH2 domain of immunoglobulin G. This domain contains the major epitopes of rheumatoid factors. This study aimed to analyze if the p205-specific T cell responses are also directed against RF epitopes and to analyze the role of p205 in the production of rheumatoid factors in general.

p205 was enriched from synovial fluid as described earlier. p205-derived peptides were chemically synthesized. T cell proliferation assays were performed with cells obtained from RA and control patients and healthy individuals.

Sequencing and mass spectrometry by matrix assisted laser desorption-time of flight (MALDI-TOF) of p205 revealed that it contains sequences with similarity and identity to IgG and other members of the immunoglobulin superfamily. p205 was detected in the synovial membrane of RA patients by antisera specific for p205-derived peptides. Cells staining positive for p205 were also positive for the macrophage marker CD68. p205 staining did never occur in B cell clusters staining positive for CD19 or in T cell infiltrates staining positive for CD3. No B and T cells were detected in the highly p205-positive lining and sublining of the synovial membrane. p205 could react with monoclonal rheumatoid factors (RF). Those RF that reacted also with p205 tended to be of a binding specificity characteristic of RA. Those RF that did not react with p205 tended to be of a binding specificity that is also observed in healthy immunized donors or patients with Waldenström's macroglobulinemia.

Synovial fluid (SF), SF-derived p205 and p205-derived peptides were used as antigens in T cell proliferation assays. As control antigens, a mock peptide and PHA were used. SF, p205 and p205-derived peptides stimulated T cells from two thirds of RA patients, but not from patients with other rheumatic diseases or from healthy individuals. SF, p205 and the 11aa p205 peptide with sequence identity to IgG were extremely high stimulators of proliferation in the majority of RA patients and were often in the range of the mitogen PHA. Two other p205-derived peptides were also stimulatory for RA-derived T cells, but to a lesser degree and at a lower frequency of patients. None of these peptides induced T cell proliferation in patients with other rheumatic diseases or healthy individuals. No reactivity was observed with the mock peptide in any of the patients. T cells specific for p205 cocultured in the presence of IgG-specific B cells induced the production of rheumatoid factors upon stimulation with cognate antigen and the 11mer peptide 3. RFs could also be induced upon immunization of rabbits with peptide 3.

p205 is a major target of autoreactive T cells in RA and appears to be a novel member of the immunoglobulin superfamily. It contains an IgG-identical stretch and p205 is targeted by RFs. The IgG-identical peptide 3 stimulates T cells such that they can provide cross-help for RF-secreting B cells *in vitro* and *in vivo*. p205 may thus likely be the trigger of RF production in RA and may thus be of pathogenic importance.

**P53****Responses of the rat immune system to arthritogenic adjuvant-oil****L Svelander\*, BC Holm\*, A Bucht\*\* and JC Lorentzen\*\****\*Department of Medicine, Unit of Rheumatology, Karolinska Institutet, Stockholm, Sweden; \*\*Department of Biomedicine, Division of NBC Defense, Defense Research Establishment, Umeå, Sweden;**†Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden*

T-cell mediated inflammatory joint diseases with similarities to rheumatoid arthritis can be triggered in arthritis-prone rat strains by intradermal injection of adjuvant-oils. The pathogenesis of oil-induced arthritis (OIA) remains elusive, and a largely unresolved question concerns how the rat immune system responds to arthritogenic oils, such as incomplete Freund's adjuvant (IFA). Here we report that IFA induces increased plasma levels of the APR (acute phase reactants) fibrinogen and AGP (a1-acid glycoprotein) already at day 4 post-injection (d.p.i.). In contrast, no early responses were detected in the joints before infiltration of T-cells, which coincided with arthritis onset at 11-14 d.p.i. The infiltrating cells were possibly derived from draining lymph nodes (LN), which contained dramatically increased cell numbers from 4 d.p.i. onwards. The magnitude of the early increase in cell numbers and APR was regulated by non-MHC genes, as determined by comparison between arthritis-susceptible DA rats and arthritis-resistant but MHC-identical LEW.1AV1 and PVG.1AV1 rats. These resistant strains had high plasma AGP at 4 d.p.i. whereas DA rats did not - possibly reflecting a deficient anti-inflammatory response in this strain. Furthermore, the relative increase in LN cell numbers was largest in DA rats, which is intriguing considering that LN T-cells can transfer arthritis. Analysis of LN after *in vivo* labelling with BrdU revealed increased numbers and proportions of proliferating lymphocytes. Furthermore, PCR-analysis of LN cytokine mRNA revealed up regulation for IL-1b at 4 d.p.i. When an immunogen (ovalbumin) was added to the adjuvant an immune response was clearly traced as increased mRNA for IL-4, IFN-g and IL-1b, and in increased numbers of proliferating lymphocyte *in vivo*.

In summary, we provide evidence that arthritogenic oil induces an early systemic inflammatory response, as well as activation of cells and lymphocytes in draining lymph nodes, but no signs of cell activation in the joints before onset of arthritis.

**P54****Characterization of autoreactive T cells to the autoantigens hnRNP-A2/RA33 and filaggrin in patients with rheumatoid arthritis and controls****R Fritsch, D Eselböck, B Jahn-Schmid, C Scheinecker, B Bohle, K Skriner, J Neumüller, J Smolen and G Steiner***Division of Rheumatology, Institute of Biochemistry, Institute of Experimental Pathology and Institute of Histology, University of Vienna, Austria*

In an attempt elucidate the role of autoimmune processes in the pathogenesis of rheumatoid arthritis (RA) we investigated the T cell responses to two autoantigens targeted by autoantibodies of patients with RA, (i) the heterogeneous nuclear ribonucleoprotein (hnRNP) A2/RA33 and (ii) filaggrin which is one of the target structures recognized by anti-citrulline antibodies.

Stimulation assays were performed with peripheral blood mononuclear cells of 50 RA patients, 20 patients with osteoarthritis and 21 healthy control individuals using recombinant hnRNP-A2/RA33 as well as some fragments thereof and recombinant filaggrin both in unmodified and citrullinated form. Antigen-specific T cell clones

(TCC) were obtained by cultivating T cell lines in the presence of antigen and IL-2 followed by limiting-dilution cloning.

Proliferative responses to hnRNP-A2/RA33 were seen in 60% of the RA patients with a mean stimulation index (SI) of  $3.5 \pm 2.8$  and were significantly higher than those observed in the control group (mean SI =  $1.7 \pm 1$ ,  $P < 0.00005$ ). There was no correlation with the presence of anti-A2/RA33 autoantibodies nor with MHC genes, although more than 60% of the responsive patients carried the shared epitope. Results obtained with recombinant fragments indicated a major T cell epitope to be located in the N-terminal first RNA binding domain of the protein. Anti-A2/RA33 specific TCC ( $n = 16$ ) derived from RA patients were almost exclusively CD4+/CD8-, whereas only 7 of 12 TCC derived from controls showed this phenotype, and secreted high amounts of IFN $\gamma$  upon antigen stimulation as did all TCC derived from controls. Proliferative responses to filaggrin in either form were seen in only 25% of the RA patients tested and did not differ from those observed in the control group indicating that filaggrin-reactive T cells do presumably not drive the autoantibody response to citrullinated antigens.

Taken together, a Th1 type autoimmune response to hnRNP-A2/RA33 was commonly observed in RA patients suggesting this nuclear protein to constitute a major T cell autoantigen which might be fuelling one of the pathological autoimmune reactions that drive the destructive processes effective in RA.

**P55****Leflunomide leads to inhibition of transendothelial migration****J Grisar, GH Stummvoll and JS Smolen***Division of Rheumatology, Department of Internal Medicine III, University of Vienna, Austria*

Leflunomide is a new disease modifying drug that is widely used in the therapy of rheumatoid arthritis (RA). The active metabolite of leflunomide A771726 leads to inhibition of dihydroorotate dehydrogenase, an enzyme necessary for pyrimidine *de-novo* synthesis. Since activated lymphocytes expand their pyrimidine pool, A771726 leads to a decrease of their proliferation. A771726 also suppresses TNF mediated nuclear factor kappaB activation. We were therefore interested if A771726 also would be capable to influence transendothelial migration (TEM) of peripheral mononuclear cells (PBMC).

We investigated the TEM of PBMC, which migrated through endothelial cell monolayers in an in-vitro model. Human umbilical vein endothelial cells (EC) were cultured to confluence on collagen gels and then incubated with human PBMC of healthy blood donors. PBMC were recollected in three groups: 1) cells that did not adhere to the endothelium, 2) cells that bound to the endothelium, 3) cells that had migrated through the endothelium, and then counted by microscope. Experiments in which PBMC as well as EC were treated with A771726 (in the absence or presence of uridine) were compared to simultaneously performed control experiments. No increased toxicity on the PBMC treated with the doses of A771726 used in our experiments, was observed.

24 hour incubation of PBMC and EC with 30 mg/ml A771726 led to a significant decrease in TEM ( $11 \pm 3$  % vs.  $6 \pm 3$  % migrated cells,  $P = 0.016$ , paired T test). Uridine partly reversed the decrease in TEM when incubating PBMC and EC with both uridine and A771726.

Our results demonstrate that leflunomide may have direct anti-inflammatory effects by inhibiting the extravasation of PBMC. These data suggest, that leflunomide, besides its influences on lymphocyte proliferation, also reduces accumulation of cells at inflammatory sites.

**P56**

**Immune response to hn and snRNP in autoimmune mice. A model for the development of lupus autoimmunity by a single initiator T helper epitope?**

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Systemic lupus erythematosus is characterised by the presence of high titers of autoantibodies reacting with various components of the small and heterogeneous nuclear ribonucleoprotein particle. It has been suggested that these antibodies are produced by an antigen-driven mechanism under the dependence of antigen-specific T cells. To investigate the role of T cell help in this process, we sought with twenty overlapping peptides the Th epitopes on the U1-70K snRNP in unprimed H-2<sup>k</sup> MRL/lpr lupus mice and immunised CBA normal mice. The peptide 131-151 was recognized by both IgG autoantibodies and CD4<sup>+</sup> T cells from 7-9 week-old MRL/lpr mice. In this test, APCs from MRL/lpr mice were required, APCs from naive CBA mice failed to stimulate CD4<sup>+</sup> cells from MRL/lpr mice. Peptide 131-151 bound both I-A<sup>k</sup> and I-E<sup>k</sup> class II molecules and favoured an IL-2 positive T cell response but not IFN- $\gamma$ , IL-6 and IL-10 secretion. Segment 131-151 is localised within the RNP80 motif and contains residues that are highly conserved in many nuclear, nucleolar and cytoplasmic RNA binding proteins. In parallel, we studied the Ab response to the A2/B1 hnRNP in different murine models of lupus, and found in residues 50-70 a major epitope recognized very early during the course of the disease by Abs from most of MRL/lpr mice. Peptide 50-70 generated in CBA/J mice an effective Th cell response with IL-2 and IFN- $\gamma$  secretion. Interestingly, this peptide also contains the highly conserved sequence present in peptide 131-151 of the 70K protein. It is possible that starting from a single Th epitope, the sequence of which is repeated in several self-proteins involved in the same complex or close cellular components, a larger, diversified Th response is generated, which extends via intra-and inter-molecular spreading of the T and B cell responses.

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**P57**

**Persistence of plasma cells in the kidneys of autoimmune NZB/W mice**

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NZB/W mice develop a disease similar to human systemic lupus erythematosus (SLE), including autoantibody production, hypergammaglobulinaemia and inflammation of the kidneys. It is known that large numbers of lymphocytes infiltrate the kidneys of these mice but the role of this organ for the production of antibodies is not clear. Here, we compare the role of bone marrow, spleen and

inflamed kidneys of NZB/W mice for the activation of B cells and for the persistence of antibody secreting cells (ASC). ASC are present in the kidneys of mice with full blown disease, as many as in the spleen and bone marrow, and 50 times more than in the kidneys of normal mice. In the kidneys, ASC are located mainly in the outer medulla, close to B- and T cell infiltrates. The specificity of the ASC in the inflamed kidneys is not restricted to self-antigens. After immunization of NZB/W mice with Ovalbumin (OVA), the antigen-specific ASC are found initially exclusively in the spleen. Weeks later, during a period of at least 3 months, OVA-specific ASC are found in stable and high numbers within the bone marrow and the kidneys of these mice, but no longer in the spleen. As determined by FACS, B cells with a germinal center phenotype (B220<sup>+</sup>/PNA<sup>+</sup>) are found only in very low numbers in the kidneys, but in high numbers in the spleen of NZB/W mice. By histology, germinal centers could not be detected in the kidneys, but in the spleen. The lack of B cell activation and the kinetics of the appearance of OVA-specific ASC suggest that in autoimmune NZB/W mice kidneys, plasma cells generated during an immune reaction in secondary lymphoid organs, later accumulate and persist, like in bone marrow. These experiments identify the inflamed kidneys of NZB/W mice as site of prime relevance for the homeostasis of plasma cells, irrespective of their specificity, suggesting that chronically inflamed tissue attracts plasma cells as such and extends the overall capacity of the body for plasma cells, allowing autoreactive plasma cells to survive for long times within the inflamed tissue and to provide exorbitant titers of antibodies locally.

**P58**

**In vivo preactivated autoreactive Th cells in healthy individuals**

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The direct analysis of autoantigen-specific Th-cells has been hampered so far by the lack of appropriate methods to directly determine their frequency or functional capabilities. We have applied a set of new techniques to directly identify and analyze autoantigen-specific T-cells in both affected and healthy people according to their effector functions (e.g. effector cytokine production) after provocation with antigen.

We have used these technologies to analyze Th-cells specific for SLE-associated autoantigens, in particular nucleosomes and the ribonucleoprotein La. Surprisingly, *in vivo* pre-activated autoantigen-specific Th-cells secreting IFN $\gamma$  and TNF $\alpha$ , could be detected not only in SLE-patients, but also in normal healthy persons, with frequencies ranging from 0.02% to 0.1%. Preactivation of these cells *in vivo* was confirmed by the fact that they expressed CD45RO but not CD45RA. Some of them had down-regulated expression of CD45RB and CD27. We also detected in healthy donors *in vivo* preactivated Th-cell specific for the self-antigen alphaB-Cristallin, a small heat shock protein. Up to 0.5% of peripheral Th cells specifically react with IFN $\gamma$  secretion upon short term stimulation, a hallmark of a recall response, i.e. *in vivo* preactivation.

The fact that *in vivo* pre-activated, autoantigen-specific Th-cells can be detected at comparable frequencies and with similar cytokine secretion patterns in blood of normal persons and patients suffering from a disease in which such Th cells are suspected to play a pivotal role, points to mechanisms other than central and peripheral tolerance that control the initiation of those autoimmune reactions.

P59

### Characterization of RA33 (hnRNP-A2/B1)-autoreactive T cells in SLE-patients

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SLE is a systemic autoimmune disease with distinct immunological characteristics including defective T cell functions, especially concerning IL2 production and proliferation. Furthermore, B-cell hyperactivity is observed leading to the formation of several characteristic autoantibodies (ab), among them ab to the heterogenous nuclear ribonucleoprotein A2/B1 (hnRNP/RA33). These antibodies are known to occur in over 20% of SLE patients.

In order to elucidate the role of T cells and their influence in antibody production in SLE, we studied proliferation of PBMC to purified hnRNP-A2/B1 in 34 SLE patients and 21 healthy controls.

While the stimulation indices (SI) in the healthy control group ranged from 0.5 to 3.5 (mean SI:  $1.5 \pm 0.9$ ), the proliferative response of PBMC of the patient group ranged from 0.7 to 17 with a mean SI of  $4.8 \pm 4.0$  (only 6 of 34 patients had an  $SI < 2$ ;  $P < 0.00004$ ).

We then proceeded to draw RA33-specific T cell clones (TCC) by cultivation and limiting-dilution cloning of T cell lines. The generated 30 TCC derived from SLE patients and 19 TCC from healthy controls did not reveal a significant difference in SI and produced either more IFN $\gamma$  than IL4 or none of these cytokines at all, suggesting that these TCC were of T1 or T0, but not T2 phenotype. Interestingly though, while only 11% of healthy control patients showed a CD4-/CD8+ subtype and 16% displayed a CD4+/CD8+ phenotype, 37% of TCC derived from SLE patients were CD4-/CD8+ (and 20% expressed CD4 as well as CD8).

Our data reveal that more than 80% of SLE-patients have a significant T cell reactivity ( $SI \geq 2$ ) to the nuclear protein hnRNP-A2/B1 indicating that the antibody response might be T cell driven. Furthermore, almost 60% of TCC derived from SLE patients were CD8+, which supports the importance of these T cells in SLE. Further studies will have to elucidate the pathogenetic implications of these findings.

P60

### Signalling via T cell receptor (TCR) in patients with SLE

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**Aim:** Dominating defects in SLE are demonstrated in humoral immune response, however, various T cell defects were observed. Recently, some authors referred to about the abnormalities in signalling after TCR activation and about CD3 $\zeta$  chain deficiency of some SLE patients (1,2,3). The reports differed in many details and, therefore, we decided systematically to test these results and to study molecular architecture of such signalling complex.

**Methods:** Isolated peripheral blood lymphocytes (PBL) from SLE patients and controls were analyzed for the presence of CD3 $\zeta$  chain using immunoblot assay with mouse monoclonal anti-CD3 $\zeta$  chain antibody and secondary antibody conjugated with peroxidase fol-

lowed by chemiluminescence. Lysis of PBL was performed either following the method of American authors (1,2) or by the method used in the laboratories studying the molecular aspects of TCR signalization (e.g. Dr. V. Hořejší, Prague). T cell signalization was stimulated by cross-linking TCR with monoclonal antibody against CD3 $\zeta$  (MEM-92) and tyrosine phosphorylated proteins were detected using monoclonal antibodies (P-TYR1 and P-TYR2) by immunoblotting.

**Results:** The defect of CD3 $\zeta$  chain was found in 17 of 45 SLE patients (38%) using the protocols published by American group and was never found in 15 controls (healthy or not SLE patients). But, we could not find this defect using the improved protocol in 59 SLE patients, including all of the previous group. Signalling was different in patients compared to controls in that unstimulated cells from patients showed the pattern observed in stimulated controls but the results were dependent on the conditions by which PBL were brought to the so-called "inactive" or quiet state.

**Conclusion:** Conflicting results show that the published CD3 $\zeta$  chain deficiency in SLE patients could be caused by the methodical approach. Defect in signalization must be defined more precisely under strictly controlled conditions.

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P61

### Impaired T-cell response to subsequent TCR-stimulation after anti-CD3 induced proliferation

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**Introduction:** Defects of T-cell (TC) proliferation and in TC-receptor (TCR) signaling have been demonstrated in several autoimmune diseases. The detailed mechanisms governing activation and proliferation of activated TC, however, are still not completely known. Here, we will show that under certain conditions human peripheral blood (PB) TC, once activated by anti-CD3 monoclonal antibody (mab) *in vitro*, fail to respond to a subsequent re-stimulation via the TCR. This unresponsiveness is caused at the transcriptional level by an impaired production of IL-2, and this defect is temporary.

**Methods:** PB mononuclear cells (MC) from healthy donors were pre-stimulated (PS) by anti-CD3PS for 96 h following restimulation by IOT-3, IL-2, or IL-15, as well as other mitogens. In control experiments (NS), PBMC were cultured in medium alone for the first 4 days. Restimulation of both populations was also performed in the presence of freshly isolated monocytes (MO). Furthermore, after the first incubation T-cells and MO from PS and NS cultures were separated by magnetic beads and incubated for re-stimulation in a criss-cross design. Surface immunophenotype of both, TC and MO were analysed by flow cytometry. Cytokine production was determined by rtPCR and intracellular signaling protein content of TC in PS and NS cultures were compared by western blotting.

**Results:** PBMC pre-incubated for 96 h with medium alone showed a good proliferation to subsequent stimulation with anti-CD3 mab, whereas IL-2 induced only little proliferation. Unresponsive TC fail to produce IL-2 as demonstrated at transcription level by rt-PCR. In contrast, PS cells responded only minimally to subsequent stimulation with anti-CD3, but the addition of IL-2 induced a strong proliferation, comparable to IL-15. Both, PS and NS TC responded well to re-stimulation by PHA, whereas Con A induced proliferation mainly of NS cells and thus had similar effects as anti-CD3. In the presence of 10% freshly isolated MO PS cells were able to respond significantly to subsequent TCR challenge. But the addition of MO from NS cultures to PS-TC did not fully restore proliferation. Interestingly, when cells were allowed to rest for 168 h, the responsiveness of PS lymphocytes was restored. Surprisingly, immunoblots revealed that PS cells had a higher intracellular content of  $\zeta$ -chain and p56lck. Both, PS TC and MO show higher expression of different activation associated surface molecules (HLA-DR, CD25, CD69, and costimulatory molecules).

**Discussion:** Our results show a mechanism leading to a temporary unresponsiveness to TCR ligation of preactivated TC although adequate costimulatory support seems available. The rate limiting events for IL-2 production can be overcome by bypassing the TCR via mitogens or addition of freshly isolated MO. Although we have not been able to fully define the rate limiting events we have been able to exclude various possibilities. TC pre-activated via the TCR can continue to produce and express a variety of molecules such as IFN- $\gamma$ , IL-2R, and cell surface molecules. Thus, their effector function in G1-phase, but not their progression into a mitotic cell cycle seems to be sustained.

## P62

### Effect of CD154-CD40 interactions on collagen production by fibroblasts

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Interactions of T cells and fibroblasts appear to be important in the development of fibrosis, for example, the restrictive lung disease that follows the inflammatory process of alveolitis in scleroderma (systemic sclerosis, SSc). The intermolecular interactions mediating fibroblast activation are not well characterized. CD154 (CD40 ligand) is an activation-induced T-cell surface molecule which counter-receptor is CD40 expressed on target cells, including fibroblasts. We have found CD154 expression on a number of activated CD8<sup>+</sup> T cell clones derived from bronchoalveolar lavage (BAL) fluids from SSc patients. To begin investigating the potential role of CD154-CD40 interactions in fibroblast activation, we co-cultured CD154<sup>+</sup> Jurkat D1.1 cells or CD154<sup>-</sup> Jurkat E6-1 cells with fibroblast lines derived from dermal biopsies or BAL fluids from SSc patients and control donors. Collagen  $\alpha 2(I)$  mRNA expression in fibroblasts was measured by RT-PCR, with ribosomal protein S9 as an internal standard. Total soluble collagen was measured in co-culture supernatants, using Sircol Bicolor assay. In fibroblasts co-cultured for 6 h with CD154<sup>+</sup> cells, but not CD154<sup>-</sup> cells, normalized collagen mRNA expression and total soluble collagen production were 2 times higher than in fibroblasts cultured alone. Intracellular fluorescent staining did not detect IL-4, IL-10, IFN $\gamma$ , or CD95 ligand expression in either D1.1 or E6-1 cells. These data suggest that CD154-CD40 interactions may enhance collagen production in fibroblasts. As this process continues uncontrolled, it may lead to the development of fibrosis.

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## P63

### Enhanced transendothelial *in vitro* migration of scleroderma lymphocytes

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**Objective:** T lymphocytes are thought to play an important role in the pathogenesis of Systemic Sclerosis (SSc). Perivascular accumulations of predominantly CD4<sup>+</sup> T-lymphocytes are found at an early stage of scleroderma skin lesions. Moreover, soluble and membrane-bound adhesion molecules are elevated in SSc and may facilitate lymphocyte/endothelial cell contact. To assess the migration qualities of peripheral lymphocytes, we investigated the *in vitro* migration of SSc-lymphocytes through human endothelial cell monolayers.

**Patients and Methods:** Endothelial monolayers were formed by human umbilical vein endothelial cells (HUVEC) in their 3rd to 4th passage seeded on collagen gels and incubated overnight. PBMC were prepared from 6 patients (5f, 1m, mean age 55 $\pm$ 6.5 yr.) fulfilling the ACR criteria for SSc and 6 healthy controls (HC; 5f, 1m, mean age 55 $\pm$ 7.1 yr.). Lymphocyte-migration was measured after one hour of incubation by fractionated harvest of non-adherent, bound, and migrated lymphocytes. Changes in the CD4/CD8 ratio and in the lymphocytic expression of activation markers (CD25, HLA-DR, CD69) and adhesion molecules (CD11a, CD49d) *ex vivo*, during and after migration were investigated by fluorocytometry.

**Results:** The percentage of migrated SSc lymphocytes was increased in each single experiment (Fisher's exact test  $P < 0.03$ ) when compared to HC (9.0 $\pm$ 4.4% vs 5.3 $\pm$ 2.9%). Compared to HC, the CD4/CD8 ratio was only slightly higher in SSc when detected *ex vivo* (2.71 $\pm$ 0.76 vs. 2.22 $\pm$ 0.54,  $P = n.s.$ ), but increased after migration (3.00 $\pm$ 0.57 vs. 1.01 $\pm$ 0.38,  $P < 0.02$ ), whereas the CD4/CD8 ratio in HC fell. The expression of lymphocytic activation markers and adhesion molecules was similar in SSc and HC *ex vivo*. Migrated SSc lymphocytes tended to express higher amounts of CD 25 and CD 49d, but this did not reach statistical significance in our small sample of patients.

**Discussion:** Lymphocyte migration through a human endothelial monolayer is increased in SSc and is accompanied by an increase of the CD4/CD8 ratio. These data suggest that CD4<sup>+</sup> SSc cells are more prone to migration than CD8<sup>+</sup> cells and are in line with the paravascular accumulation of CD4<sup>+</sup> lymphocytes.

## P64

### Autologous dendritic cells stimulate human HSP60 responsive T cells, in the absence of additional exogenous antigen

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**Background:** Animal models and clinical studies of inflammatory arthritis have shown a potentially protective role for autoreactive T lymphocytes recognising the 60 kilodalton heat shock protein (hsp60). The mechanisms of this protection have not been fully characterised. We have previously demonstrated that PBMC from healthy individuals show proliferative responses to human hsp60, and clones have been isolated that recognise the self protein. The objective of the present study was to confirm the autoreactive nature of these cells and determine whether the endogenous antigen could be effectively presented by professional antigen presenting cells.

**Methods and results:** Highly purified recombinant human hsp60 was prepared along with a non-recombinant preparation of mito-

chondrial hsp60, derived from human lymphoblastoid cells. To assess the ability of professional antigen presenting cells to present the endogenous self hsp60, autologous dendritic cells were isolated from peripheral blood by negative selection, cultured in GM-CSF and IL-4, and activated with LPS prior to use. Human hsp60 responsive T cell clones from a healthy individual were shown to proliferate in response to both the recombinant preparation and the mitochondrial preparation, thereby excluding the possibility of the clones recognising bacterial contaminants. Furthermore these clones proliferated in the presence of autologous dendritic cells, activated with LPS, in the absence of exogenous antigen. The proliferative responses to the activated dendritic cells were titratable and the data suggested a requirement for additional, presumably apoptotic, cells to also be present in the culture system.

**Conclusions:** These experiments demonstrate, at a clonal level, an autoreactive repertoire in healthy individuals responding to human hsp60. The ability of these cells to recognise autologous activated dendritic cells may provide insight into the role of such cells *in vivo*. Since activated dendritic cells and increased numbers of apoptotic cells will both be present at inflammatory foci, local expansion of potentially immunomodulatory, self hsp60 responsive T cells could occur at these sites.

## P65

### Macrophages expressing the scavenger receptor CD163: a link between immune alterations of the gut and synovial inflammation in spondyloarthritis

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**Objective:** To investigate the presence, phenotype and role of synovial macrophages in SpA by immunohistochemistry and flowcytometry.

**Results:** In the synovial lining CD68, CD163 and HLA-DR were increased in SpA versus RA; in the sublining CD163 and HLA-DR were also increased. In contrast, costimulatory molecules and dendritic cell markers were scarce in SpA versus RA synovium. Interestingly, CD 163 and CD68 were also increased in colonic lamina propria in SpA. CD163 and HLA-DR in the sublining were correlated with CRP and ESR. CD163+ macrophages expressed high levels of HLA-DR and could produce TNF-alpha but not IL-10. Anti-TNF-alpha therapy in SpA induced a decrease of CD163 in both synovial lining and sublining.

**Conclusions:** Macrophages expressing the scavenger receptor CD163 are increased in synovium and colonic mucosa in SpA, highlighting the relation between joint and gut. The correlation with inflammatory parameters, the expression of HLA-DR, the production of TNF-alpha but not IL-10 and the reduction by anti-TNF-alpha therapy support a role for CD163+ macrophages in synovial inflammation in SpA.

## P66

### Fully competent dendritic cells as inducers of T cell anergy in autoimmunity

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Mature immunologically competent dendritic cells are the most efficient antigen presenting cells, that powerfully activate T cells and initiate and sustain immune responses. Indeed, dendritic cells are

able to efficiently capture antigens, express high levels of co-stimulatory molecules and produce the combination of cytokines required to create a powerful immune response. They are also considered to be important in initiating autoimmune disease by efficiently presenting autoantigens to self-reactive T cells that, in this case, will mount a pathogenic autoimmune reaction. Triggering T cells is not a simple on-off procedure, as TCR responds to minor changes in ligand with gradations of T-cell activation and effector functions. These 'misfit' peptides have been called Altered Peptide Ligands, and have been shown to have important biological significance. Here we show that fully capable dendritic cells may present, upon natural antigen processing, a self-epitope with Altered Peptide Ligands features that can unexpectedly induce anergy in a human autoreactive T cell clone. These results indicate that presentation of a self-epitope by immunologically competent dendritic cells does not always mean 'danger' and show a novel mechanism involved in the fine balance between T cell activation and tolerance induction in man.

## P67

### Dendritic cell subsets in rheumatoid arthritis

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Distinct myeloid DC and lymphoid DC subsets have been described, which regulate the nature and magnitude of immune responses. Therefore DC function must be carefully regulated, otherwise inappropriate responses may result in such chronic inflammatory diseases as rheumatoid arthritis (RA). In this study the composition and activation state of DC subsets was compared between autologous blood, synovial fluid and synovial tissue of RA patients using 4-colour flow cytometry. Preliminary results indicated that RA blood and normal blood had a similar ratio of DC subsets, both of which exist in a relatively inactivated state. In contrast, myeloid DC were predominant in RA synovial fluid and synovial tissue. In synovial tissue these myeloid DC were more highly activated and localized to lymphoid aggregates. Lymphoid DC were scarce in both synovial fluid and synovial tissue.

**Conclusion:** These results suggest that myeloid DC play a key role in the pathogenesis of RA and supports the view that RA is predominantly a Th1-mediated disease.

## P68

### Detection of bacterial components in synovial tissue from patients with inflammatory arthritis by using PCR with pan bacterial 23S rRNA and 16S rRNA primers, and gas chromatography-mass spectrometry

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Using PCR for 16S rRNA, the presence of bacterial DNA in synovial tissue (ST) from a variety of inflammatory arthritides has been reported. To confirm this, we have applied the PCR with pan bacterial 23S rRNA and 16S rRNA primers, which both methods have been used successfully for bacterial identification in various clinical samples.

ST were collected at joint surgery from 81 patients: 42 rheumatoid arthritis (RA), 31 osteoarthritis (OA), 8 other inflammatory arthri-

tides. Extremely strict precautions were followed in the clinics and laboratory to prevent contamination. Bacterial DNA could not be detected by PCR with pan 23S rRNA and 16S rRNA in any of the samples. The positive controls, including bacterial DNA and human DNA, were run with each sample, and were always positive. Further, using the same method, 5/15 (33%) synovial fluid samples from patients with *Chlamydia* reactive arthritis were PCR positive. The PCR sensitivity was 2-20 CFU/reaction determined by mixing the living bacteria with ST and using exactly the same experimental procedure as with the patient samples.

Gas chromatography-mass spectrometry has been applied to detect muramic acid (bacterial cell wall specific chemical component) in ST. Preliminary results suggest that low concentration of muramic acid can be detected in the ST from some patients with inflammatory arthritis.

Our results show that bacterial DNA in ST from RA and OA could not be detected by PCR for 23S rRNA and 16S rRNA. Instead, muramic acid could be detected by gas chromatography-mass spectrometry. These observations indicate that the presence of bacterial DNA in ST might not be as prevalent as previously suggested. Nevertheless, the bacterial components may exist in ST.

## Poster Discussion E

### Autoantibodies in CTDs

#### P69

#### Detection of anti-B/B' UsnRNP antibodies in connective tissue disease sera by Western immunoblot

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**Introduction:** The fine antibody specificity towards protein components of uridine-enriched small nuclear ribonucleoproteins (UsnRNP) may be investigated by several methods including the Western immunoblot. Crucial in Western blot techniques' reliability is the origin and nature of the antigenic source.

**Aim:** To assess the significance of antibodies to B/B' proteins detected by Western immunoblot in connective tissue disease (CTD) patients.

**Methods:** Three hundred and forty-eight patients with well diagnosed CTD (101 SLE, 51 systemic sclerosis, 53 primary Sjogren's syndrome, 27 poly/dermatomyositis, 15 rheumatoid arthritis and 101 overlap CTD) and 31 matched healthy subjects were studied. In addition, sera from 13 patients with primary Epstein-Barr virus (EBV) infection (10 in acute primary infection and 3 with anamnestic past infection) and high titer IgG anti-EBV antibodies were tested. IgG anti-UsnRNP as well as anti-ribosomal P protein antibodies were determined by Western blotting on total Raji cell extract (a cell line transformed by EBV). Antinuclear and anti-dsDNA antibodies were detected by indirect immunofluorescence on HEP-2 cells and *Crithidia luciliae* respectively, anti-ENA by counterimmunoelectrophoresis. Statistical analysis was performed by chi-square test.

**Results:** An unsuspectedly high frequency of anti-B/B' antibodies was found, confined to SLE (54.4%) and overlap CTD with SLE

features (55.2%). Anti-B/B' antibodies were closely associated with other anti-UsnRNP antibodies ( $P < 0.0001$ ), gel precipitating anti-nRNP antibodies ( $P < 0.0001$ ) and anti-ribosomal P antibodies ( $P = 0.0013$ ). Band patterns unequivocally different from those obtained with autoimmune sera, were provided by anti-EBV positive sera. Noteworthy, a peptide with an apparent MW corresponding to that of B peptide (28kDa) was clearly recognized by 9/10 sera from active EBV infection but not by anamnestic EBV infection sera.

**Conclusions:** The Sm spliceosomal complex is one of the most important targeted autoantigens in SLE. Western immunoblot on Raji cells provides a reliably sensitive and specific antigenic source for anti-Sm B/B' antibodies. Such high immunoreactivity could be explained by the strong cross-reactive potential of B/B' proteins and not by EBV cell transformation. Further studies are in progress to comparatively evaluate the suitability of other cell lines as an antigenic source.

#### P70

#### Comparison of different methods for the detection of the fine specificity of anti-Ro/SSA response

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**Background:** the determination of the fine specificity of anti-Ro/SSA response is useful in the classification of the risk for the occurrence of congenital complete heart block (CCHB) in newborn of anti-Ro/SSA mothers.

**Aim of the study:** to evaluate different methods for the detection of anti-52 and 60 kD Ro/SSA antibodies.

**Patients and methods:** 132 sera (82 from anti-Ro/SSA patients by counterimmunoelectrophoresis (CIE), 30 from anti-ENA positive/anti-Ro/SSA negative and 20 from ANA and anti-ENA negative) were tested by ELISA with recombinant 52 and 60 kD Ro protein (Pharmacia Upjohn, Germany) and immunoblotting (IB) with human spleen extract (HSE) as substrate to the aim of determining the fine specificity of anti-Ro response. In addition, 21 sera from mothers of CCHB children were tested by CIE, two ELISAs with recombinant proteins (Pharmacia and Euro-diagnostica, The Netherlands), two IBs with HSE and with HEP-2 extract as substrates (MarDx, USA).

**Results:** the total agreement between ELISA (Pharmacia) and IB (HSE) was 76% for anti-Ro 60 kD and 44% for anti-Ro 52 kD. The ELISA was more sensitive than IB both for anti-Ro 60 kD (91% vs 84%) and for anti-Ro 52 kD detection (82% vs 51%). Seven sera positive by CIE were negative by IB (non blotters): six of these sera were positive for anti-60 kD and 2 for anti-52 kD by ELISA. The mean antibody titre for anti-60 kD was significantly lower ( $P < 0.00005$ ) than that of sera detected by IB.

A correlation ranging from 78 to 100% was detected between the different methods testing the sera from CCHB mothers. The agreement between the IB methods for anti-Ro 60 kD and for anti-52 kD was 79% and 68.5% respectively while between the ELISAs was 44% and 67% respectively. The best agreement obtained comparing IB and ELISA for anti-Ro 60 and 52 kD was 78% between IB with HSE and ELISA (Euro-diagnostica).

**Conclusions:** ELISA seems to be the most sensitive method to detect the fine specificity of anti-Ro/SSA response. The majority of IB negative/CIE positive sera (non blotters) were positive for anti-60 kD by ELISA at low titer. IB with HSE as substrate performed slightly better (p not significant) than IB with HEP-2 cells extract in CCHB mothers.



**P71****Study of the anti-idiotypic response to anti-LA/SSB autoantibodies using complementary peptides to B- and T-cell epitopes of La/SSB****JG Routsias\*, E Touloupi\*, A Mouliat† M Sakarellos-Daitsiotis†  
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Autoantibodies to La/SSB are found in sera of patients with primary Sjogren's Syndrome (pSS) and Systemic Lupus Erythematosus (SLE). A large body of these autoantibodies are directed towards discrete linear epitopes comprising the sequences 289-308 aa and 349-364 aa. Several previous studies have shown that in the sequence 289-308 aa resides also a T-cell epitope. Based on "molecular recognition" theory, complementary peptides cpep289-308 and cpep349-364, derived by anti-parallel readings of the non-coding strand of La/SSB DNA encoding epitopes 289-308 and 349-364 respectively, were synthesized. Complementary peptides cpep289-308 and cpep349-364 presented inverted hydrophobicity profiles, compared with sense peptides and recognized by 28% and 51% of anti-La/SSB positive sera respectively. F(ab')<sub>2</sub> fragments were prepared after pepsin enzymatic degradation of affinity purified anti-pep and anti-cpep specific IgG. Anti-pep IgG found to specifically recognize anti-cpep F(ab')<sub>2</sub> fragment and vice versa, suggesting an idio-anti-idiotype relation. Homologous inhibition with soluble anti-pep or anti-cpep F(ab')<sub>2</sub> further confirmed this relation. In addition soluble pep, cpep or recombinant La/SSB inhibited (65%-85%) anti-pep and anti-cpep interaction indicating that the idio-epitope is located within the antigen binding site of anti-La/SSB antibodies. Anti-pep349-364 antibodies, purified from different patient sera were all found to recognize the same anti-cpep F(ab')<sub>2</sub> suggesting that a common idio-epitope exists. Immunizations of BALB-c non-autoimmune mice with pep289-308 produced anti-pep289-308 followed by the production of anti-cpep289-308 antibodies 10 days later. In a similar manner immunization with cpep289-308 led to the appearance of anti-cpep289-308 followed by the formation of anti-pep289-308 antibodies. In conclusion, antibodies to B-cell epitopes of La/SSB contain in their antigen binding site a common idio-epitope which can be detected using complementary peptides to these epitopes. Antibodies to La/SSB epitopes are target of an anti-idiotypic response against this common idio-epitope. Manipulation of this Id - anti-Id network may provide potential insights into the understanding of the molecular mechanisms for autoantibody production and therapeutic approaches.

**P72****Induction of immune responses in inbred mice by immunizations with the complementary 289-308 La/SSB epitope****Á Dotsika\*, I Papamatheou\*, P Tsagozis\*, Á Éaragouni\*,  
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It has been recently reported a methodology of manipulating antibody and T cell-mediated autoimmune responses via activation of anti-idiotypic and/or anti-clonotypic networks. This methodology was based on immunization with the complementary peptide against antigen receptors on epitope-specific B and T cells. The region 289-308 of the La/SSB protein is one of the four linear B

cell epitopes which is recognized by sera from patients with primary Sjogren's Syndrome (pSS) and by different predictive methods share also putative T cell epitope properties. The 289-308 epitope (denoted Po25) and its complementary form encoded by complementary RNA (denoted Pcp125) were conjugated on Sequential Oligopeptide Carrier (SOCn). SOCn is formed by the (Lys-Aib-Gly)<sub>n</sub> sequential motif, where  $n = 2-7$ , and the peptide antigens were anchored to the lysine groups so as they retain their original structure and they obtain favorable molecular recognition conformations. Different doses of peptide carrier formulations were administered alone or together with Freud's adjuvant (CFA/IFC) and specific antibody and lymphoproliferative response were determined. Both the *in vivo* and *in vitro* responses were dose dependent and a demonstrable cross reactivity was observed at the T and the B cell level. Immunization with Po25-SOC resulted to the induction of anti-Pcp125-antibodies and immunization with Pcp125 led to the production of anti-Po25 response indicating the regulatory activity of anti-idiotypic antibodies. On the other hand immunized mice also primed specific T cell proliferative responses in spleen. The ability of complementary peptides to prime both anti-idiotypic and T lymphocyte responses may be central to their potent immunization properties in regulating autoreactive B and T cells. With this approach we aim to shed light to the immunoregulatory mechanism(s) which underline the autoimmune response.

**P73****Structure and function of an autoantigen, alpha-enolase****S Moscato, F Bongiorno, F Pratesi, M Scavuzzo, S Bombardieri  
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The glycolytic enzyme 2-phosphoglyceratelyase (alpha-enolase) is an autoantigen in connective tissue disorders, and more frequently in patients with active renal disease. The enzyme has pleiotropic functions: it is also a structural protein, a stress protein induced by hypoxia and it acts as transcription factor in the nucleus. Alpha enolase is encoded by a single copy gene and only one mRNA species is detected. In order to define a structural basis for these different functions, we analyzed the isoelectric point of the enzyme. On a kidney extract fractionated by 2D electrophoresis, a mouse anti-enolase antiserum detects 5 spots of identical molecular weight but differing in pI. Some autoimmune sera react with all the spots, while other recognize only the acidic forms of alpha-enolase. We then analyzed the properties of the membrane form of enolase. Enolase is not a membrane structural protein, but it is strongly associated with the membrane, where it acts as plasminogen receptor. Anti-enolase antibodies purified from autoimmune sera react also with the membrane form of alpha-enolase: by flow cytometry, 7/9 antibody preparations bind in fact U937 cells, a human lymphomonocytoid cell line that expresses high density of plasminogen receptors. To investigate the possible functional role of membrane enolase, we evaluated the ability of monoclonal anti-enolase antibodies to induce cell damage or apoptosis. No monoclonal had a cytotoxic effect on U937 cells or was able to induce apoptosis in the same cell line. We then tested the ability of monoclonal anti-enolase antibodies to induce Ca<sup>2+</sup> influx in U937 cells. One out of 4 monoclonal antibodies induced release of Ca<sup>2+</sup> from intracellular stores.

In conclusion, alpha-enolase exists as multiple isoforms, probably due to posttranslational modifications, which seem to affect recognition by autoantibodies. It is presently unknown whether these modifications are tissue-specific and/or affect membrane expression of the enzyme. A possible link between Ca<sup>2+</sup> influx and receptor functions of enolase is currently under investigation.

## P74

### Recombinant anti-P proteins antibodies isolated from human autoimmune library: reactivity, specificity and epitope recognition

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**Introduction:** The ribosomal phosphoproteins P0, P1 and P2 are targeted by autoantibodies in SLE. The presence in the patient sera of the anti-P antibodies is highly specific for the disease and correlates with psychiatric, renal and liver involvement. In order to better characterize these autoantibodies (reactivity, specificity and epitope recognition), recombinant anti-P monoclonal antibodies were isolated from a human SLE patient derived phage display library.

**Methods:** Two synthetic peptides were used to select the recombinant anti-P antibody fragments: a synthetic peptide representing the C-22 common immunogenic region of the three P proteins and the multiple antigenic peptide (MAP) carrying four copies of the last 13 residues of the C-22. The human library was derived from the bone marrow lymphocytes of an anti-P positive SLE patient. The selected anti-P antibodies were tested for reactivity with the C-22, the MAP and a control panel of recombinant autoantigens in an ELISA assay. Specificity of the selected antibodies was further analyzed by immunoblotting and immunoprecipitation assays using Jurkat total cell extract. Indirect immunofluorescence staining on HEP-2 cells was also performed. Using different synthetic peptides derived from the C-22 peptide epitope recognition was further characterized in an ELISA assay. Sequencing of the selected antibody fragments was performed and the antibody sequence was compared to the nearest germ-line sequence. In all the experiments human anti-P positive control sera were included.

**Results:** Six recombinant anti-P antibodies were isolated from the human library when using the C-22 synthetic peptide. Some of the isolated antibodies reacted specifically with the C-22 antigen in ELISA, others recognized the ribosomal P proteins on Western blot, immunoprecipitated the P proteins from the Jurkat cell extract and showed cytoplasmic staining on HEP-2 cells in an immunofluorescence assay. The selected antibodies exhibited features similar to serum antibodies of the patients with respect to their reactivity, specificity and epitope recognition.

**Conclusions:** The phage display technology proves once again to be a very useful technique for the production of human monoclonal autoantibodies and for the characterization of the reactivity and specificity of these autoantibodies.

## P75

### Dominance of hydrophobic reading frames in complementarity determining region 3 of variable heavy chain genes from a patient with untreated SLE

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of multiple autoantibodies (AAb), especially anti-dsDNA Ab. It is not known whether an aberrant V(D)J

recombination process itself predisposes to the generation of autoreactive Ab, or whether abnormalities in selection influences can lead to the generation of AAb. Immunoglobulin (Ig) heavy (H) and Ig light chains of an antibody are generated from variable (V), diversity (D) and joining (J) gene segments through V(D)J rearrangements. Diversification mechanisms inherent to the rearrangement reaction ensure that D elements can potentially be used in all reading frames (RF). In addition, D and J elements of the IgH chains encode the complementarity determining region (CDR) 3 that constitutes a significant part of the Ig antigen binding site. Since it has been suggested that the CDR3 of Ab in SLE is different from that found in normals, we compared the CDR3 obtained from Ab of an untreated SLE patient with that from normal individuals. D segments of Ab from normal donors are preferentially used in RF II (26/48, p\* 0.001) that most often encodes hydrophilic antibodies. Comparison of productive and nonproductive rearrangements suggests, that this is the result of the recombinational process rather than selection. In contrast, RF II was significantly less often used in SLE Ab (4/17, p\* 0.03). In both, normal and SLE Ab, D segments were significantly less often found utilizing RF that encode stop codons. Similar to the usage of RF II, in normals this seems to be the result of the recombination process rather than selection. Because of the low number of nonproductive rearrangements in the SLE analysis it is not possible to estimate whether this results from selection or the recombination process. In contrast to the analysis of the RF, no significant difference between the length or composition of the CDR3 from SLE and normal Ab was found.

## P76

### Comparative analysis of anti-histone and anti-chromatin antibody specificity in lupus erythematosus cell-positive and -negative sera and their relation to disease activity

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Anti-histone and anti-chromatin antibody responses play a central role in the autoimmune response of systemic lupus erythematosus (SLE). Furthermore, anti-histone H1 antibodies are essential for the formation of the lupus erythematosus cell (LEC) phenomenon. In this study, the binding properties of LEC+ and LEC- SLE sera to chromatin-associated nuclear antigens (histones H1, H2A, H2B, H3, H4; complexes of H2A-H2B, [H2A-H2B]-DNA, H1-DNA; total and H1-stripped chromatin; native and denatured DNA) were investigated. In addition, sera from patients with drug-induced lupus (by procainamide, hydralazine, or quinidine), as well as from patients with rheumatoid arthritis and osteoarthritis, were assessed. Enzyme-linked immunosorbent assay was used to detect specific antibody binding. Mirroring the important role of histone H1 in the formation of LE cells, anti-histone H1 reactivity was 8-fold higher in LEC+ sera than in LEC- sera. In addition, reactivities to most of the other antigens tested, i.e., other histones and histone-DNA complexes as well as chromatin and DNA, were significantly higher in LEC+ sera than in LEC- sera. All but 1 serum sample from the patients with drug-induced lupus were negative for LE cell formation as well as for anti-histone H1 reactivity, but displayed high antibody reactivities to histone-DNA complexes, including chromatin. Sera from patients with rheumatoid arthritis and osteoarthritis did not show significant binding to these antigens. When comparing the clinical features of LEC+ and LEC- SLE patients, severe organ involvement, including nephritis and

central nervous system involvement, was common in the LEC+ group, but rare in the LEC- group. A positive LE cell phenomenon not only correlated with the presence of high anti-histone H1 antibody levels in SLE, but also indicated serologically and clinically active disease with major organ involvement.

## P77

### Immuno-serological profile of Systemic Erythemic Lupus (SLE) patients with neuropsychiatric manifestations (NP)

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**Introduction:** goal of the study was to establish the correlation between immuno-serological anomalies and the existence of NP in patients with SLE

**Methods:** the study included 60 SLE patients (54 female, 6 male), all with signs of NP. The age of patients varied between 17 and 71 years (42.3 + 13.0). The study consisted of clinical evaluation by rheumatologist, neurologist, psychiatrist, neuro-ophthalmologist, as well as electrophysiological (EEG, EP, EMNG) methods. It also included visualization (NMR) methods for determining pathologies in the CNS. Different auto-antibodies and other immuno-serological markers displayed positive results with the following frequency: ANA (91.7%); anti-dsDNA (40.0%); anti-DNP (48.3%); CIK (45.0%); low C3/C4 levels (18.3%). Antiphospholipid antibodies (aPL) were positive: LA in 14.8% pts; aCL (IgG and IgM) in 23.1% pts. 30 patients were tested for anti-SM, anti-U1RNP, cANCA and pANCA and were positive in: anti-SM- 20.0%, anti-U1RNP- 50.0%, cANCA- 10.0%, pANCA- 30.0%.

**Results:** there was no statistically significant correlation between ANA, anti-dsDNA, CIK, anti-DNP, C3/C4 and the diagnosis of certain neuro-psychiatric manifestations in our SLE patient group. Patients with focal cerebral dysfunctions were shown to have a higher frequency of aPL: LA ( $P = 0.0111$ ) and aCL ( $P = 0.0148$ ). A correlation was found between cANCA ( $P = 0.0406$ ), pANCA ( $P = 0.0348$ ), anti-U1RNP ( $P = 0.0309$ ) and skin vasculitis, as well as between pANCA and CVI diagnosis in neuro-lupus patients ( $P = 0.0028$ ).

**Conclusion:** our study did not show the correlation between auto-antibodies in SLE patients with NP and certain types of CNS/PNS lesions, except for the connection between aPL and focal cerebral dysfunctions, as well as pANCA's correlation with cerebro-vascular stroke diagnosis.

## P78

### The infectious origin of the antiphospholipid syndrome: induction by passive transfer of anti-β2GPI Abs induced by common bacteria

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The antiphospholipid syndrome is characterized by a wide variety of vaso-occlusive manifestations associated with autoantibodies

directed against β2-glycoprotein-I. The pathogenicity of anti-β2GPI antibodies has been demonstrated in animal models. The factor(s) causing production of anti-β2GPI remain unidentified, but several indirect arguments support the idea that microbial agents might influence the course of antiphospholipid syndrome and an association with microbial pathogens has recently been documented. Microbes can contain chemical structures that mimic normal host self-proteins, a phenomenon termed *molecular mimicry*. Employing a peptide phage display library, we identified hexapeptides that react specifically with anti-β2GPI monoclonal antibodies. These peptides were found to modulate the experimental model for antiphospholipid syndrome. In the current study we found high homology between one of the hexapeptides- TLRVYK, and various bacteria and viruses. We therefore immunized naive mice with a panel of TLRVYK-corresponding microbial particles to find whether they possess a pathogenic potential for autoimmunity. We show that mice which were infused with antibodies derived from mice immunized with tetanus toxoid, haemophilus influenzae or with neisseria gonorrhoeae developed clinical manifestations of experimental antiphospholipid syndrome (e.g. mice infused with anti-β2GPI derived from tetanus immunized developed thrombocytopenia  $497 \pm 98 \times 10^3$  cells/dl compared to  $1012 \pm 214 \times 10^3$  cells/dl in control mice, high percentage of fetal resorption  $48 \pm 3\%$  in comparison to  $4 \pm 2\%$  and prolonged aPTT  $69 \pm 4$  sec in comparison to  $23 \pm 3$  sec in mice infused with IgG from Shigella dysenteriae immunized mice). The pathogenetic mechanism for anti-β2GPI generation seems to be an epitope mimicry with common bacterial molecules.

## P79

### Impaired *in vitro* thrombin generation in β2-glycoprotein I null mice

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β2-glycoprotein I (β2GPI) is a target antigen for 'antiphospholipid' antibodies. These antibodies are of considerable clinical importance because of their strong association with thrombosis, recurrent fetal loss, and thrombocytopenia. Although β2GPI has been shown to have a number of anticoagulant properties *in vitro*, its role *in vivo* is unknown. The aim of this study was to evaluate the function of β2GPI *in vivo* using a β2GPI deficient mouse model. We employed homologous recombination to disrupt the β2GPI gene in embryonic stem cells, which led to the generation of mice deficient in β2GPI. To confirm that the appropriate gene was targeted, nucleotide sequencing, map location, Northern blot analysis and Western blot analysis of the expected protein was performed. Following heterozygous (+/-) intercrosses, a total of 336 surviving offspring were genotyped. Interestingly, only 8.9% of these offspring were homozygous (-/-) for the disrupted allele, suggesting an effect on embryonic implantation or development. The remaining β2GPI-/- mice progressed normally to term and the adult mice appeared to be normal by anatomical and histological analysis. However, *in vitro* thrombin generation using a novel chromogenic assay demonstrated that there was a marked decrease in thrombin generation in -/- (OD405=0.175) compared to +/- (OD405=0.312) and +/+ (OD405=0.576) ( $n = 10$ ). This would indicate that β2GPI is likely to have a prothrombotic role *in vivo*. This finding is in contrast to results obtained in *in vitro* assay system using purified β2GPI which demonstrate anticoagulant activity for β2GPI. These knockout mice also provide a valuable *in vivo* model system for exploring the role of β2GPI in disease pathogenesis.

**P80**

**Improving an anti-β<sub>2</sub>GPI ELISA by reducing the influence of a blocking agent**

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There are still considerable interlaboratory differences in positivity rate in anti-β<sub>2</sub>GPI ELISA. We have already shown that BSA as a blocking agent could introduce a substantial interference effect in an anti-β<sub>2</sub>GPI ELISA.

The aim of this study was to validate and possibly reduce an interference effect of different blocking agents on the detection of IgG anti-β<sub>2</sub>GPI antibodies by ELISA.

We used Costar high binding plates coated with affinity purified human β<sub>2</sub>GPI and blocked with 1% BSA or 3% gelatin in PBS. Selected sera (20 NHS, 20 APS sera and 10 sera from children with atopic diseases) were diluted in PBS containing 0.05% Tween (PBS-T) or in 0.1% BSA/PBS-T or in 1% gelatin/PBS-T.

When plates were blocked with BSA and samples diluted in PBS-T, 11/50 sera expressed values above the cut-off level in the wells coated with β<sub>2</sub>GPI and also substantial binding in sample blanks wells (SB) mostly exceeding the binding to the antigen, therefore these samples were considered negative (average SB for all sera =  $x \pm SD = 63 \pm 127$  mOD). The specificity of IgG antibodies yielding high background bindings was confirmed by direct binding to BSA on solid phase (correlation with SB:  $P < 0.001$ ,  $R^2 = 0.88$ ) and efficient inhibition by fluid phase BSA. Further, the sera were diluted in 0.1% BSA/PBS-T, which resulted in negligible binding to BSA either directly coated on the plates or used as the blocking agent and hence lowered SB to insignificant levels (SB =  $13 \pm 9$  mOD). Following this modification, 3/11 sera previously found negative due to high SB values, clearly expressed low positive IgG anti-β<sub>2</sub>GPI values. The inhibition of anti-BSA with 0.1% BSA in fluid phase was almost complete in 3 minutes, suggesting that longer preincubation time may be unnecessary.

1% gelatin/PBS-T as the sample diluent buffer did not prevent the substantial binding to BSA used as the blocking agent either (SB for 20 sera with the highest binding to BSA =  $203 \pm 262$  mOD). The same was true even when the plates were blocked with 3% gelatin and samples diluted either in PBS-T (SB =  $117 \pm 120$  mOD) or in 0.1% BSA/PBS-T (SB =  $127 \pm 122$  mOD) generating substantial SB values in 18/38 tested sera. Similarly to BSA, significantly lower background bindings were reached only when gelatin was used as the blocking agent and 1% gelatin added to the sample diluent buffer (SB =  $30 \pm 21$  mOD).

To reduce the interference effects of a blocking agent it was essential to dilute sera in a buffer containing the same agent. Since the binding to BSA or gelatin was detected in both normal human and patients' sera we suggest to follow this general guideline in anti-β<sub>2</sub>GPI ELISA to better define the cut-off points and to more accurately verify not only high, but also most of low positive results.

**P81**

**Heterogeneous behaviour of anti-β<sub>2</sub>-glycoprotein I antibodies on different "high binding" microtiter plates**

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We recently identified anti-β<sub>2</sub>GPI antibodies in a high proportion of sera from children with atopic dermatitis (AD) and showed that

these anti-β<sub>2</sub>GPI most probably recognise domain V of β<sub>2</sub>GPI by contrast to anti-β<sub>2</sub>GPI from patients with the anti-phospholipid syndrome (APS) which epitopes apparently reside in domain I or IV.

The aim of the present study was to compare the binding of IgG anti-β<sub>2</sub>GPI in AD and APS on four representative commercially available types of high binding microtiter plates.

Selected plates: Costar, Nunc, Linbro and Sumilon C. Randomly selected sera from 29 children with AD and sera from 43 SLE patients (24 with secondary APS) were tested by anti-β<sub>2</sub>GPI ELISA using affinity purified β<sub>2</sub>GPI. Assays were calibrated with the HCAL, chimeric anti-β<sub>2</sub>GPI monoclonal antibodies with human γ1 constant regions.

The calibration curves for HCAL were practically the same on all four types of plates. Sera from 7/24 APS patients with medium or high anti-β<sub>2</sub>GPI levels showed similar binding properties on all four plates, while 3/24 sera expressed values either slightly above or below the cut-off points. On the other hand, anti-β<sub>2</sub>GPI from AD sera showed very similar binding on Costar, Nunc and Linbro plates, while only 3/13 positive sera with the highest values on these 3 types of plates expressed low positive values for IgG anti-β<sub>2</sub>GPI on Sumilon C plates (Table 1). Except for one serum (low positive on Linbro plate) all sera from SLE patients without APS were negative on all the plates.

Our results point to substantial differences in the binding to β<sub>2</sub>GPI coated on different microtiter plates by anti-β<sub>2</sub>GPI in AD (with no signs of APS) but not by anti-β<sub>2</sub>GPI in APS. In contrast to the other plate types, Sumilon C plates coated with β<sub>2</sub>GPI bound only minimally antibodies from AD children. If such anti-β<sub>2</sub>GPI prove non-thrombogenic, we will be able to increase the specificity of detecting anti-β<sub>2</sub>GPI relevant for APS by the use of this type of microtiter plates. Alternatively, if both anti-β<sub>2</sub>GPI specificities prove thrombogenic, we will be able to increase the sensitivity of the assay system by the use of other less discriminatory types of plates.

Table 1 COSTAR NUNC LINBRO SUMILON C

|              | k    |      | R <sup>2</sup> |      | N    |    | k    |      | R <sup>2</sup> |       | N    |    | k |  | R <sup>2</sup> |  | N |  |
|--------------|------|------|----------------|------|------|----|------|------|----------------|-------|------|----|---|--|----------------|--|---|--|
| APS (n = 24) | 1.00 | 1.00 | 8              | 1.06 | 0.99 | 8  | 1.09 | 0.95 | 9              | 0.84  | 0.99 | 9  |   |  |                |  |   |  |
| AD (n = 29)  | 1.00 | 1.00 | 13             | 1.05 | 0.97 | 13 | 0.87 | 0.92 | 13             | 0.22* | 0.63 | 3* |   |  |                |  |   |  |

k, slope of linear regression plot and R<sup>2</sup> - determination coefficient: both compared to Costar. N, number of IgG anti-β<sub>2</sub>GPI positive sera in the group. \*P < 0.001 (significant difference when compared with Costar, Nunc or Linbro)

**P82**

**Oxidation of β<sub>2</sub>-glycoprotein I (β<sub>2</sub>GPI) by the hydroxyl radical alters phospholipid binding and modulates recognition by anti-β<sub>2</sub>GPI autoantibodies**

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We investigated whether β<sub>2</sub>GPI, the key antigen in the antiphospholipid syndrome, is susceptible to oxidative modifications by the hydroxyl radical (°OH) that may influence its lipid-binding and antigenic properties. We compared the effects on human and bovine β<sub>2</sub>GPI of °OH free radicals generated by γ-radiolysis of water with <sup>137</sup>Cs and by the Fenton system composed of Fe-EDTA, ascorbate and H<sub>2</sub>O<sub>2</sub>. Radiolytic °OH caused a dose-dependent loss of tryptophan, production of di-tyrosine and carbonyl groups, dimerization

and/or extensive aggregation of  $\beta_2$ GPI. It ensued a reduction in affinity binding to cardiolipin liposomes and loss of  $\beta_2$ GPI-dependent autoantibody binding to immobilized cardiolipin. Patient anti- $\beta_2$ GPI antibodies segregated into two groups based on the effect in the  $\beta_2$ GPI-ELISA of  $\beta_2$ GPI pretreatment with radiolytic  $^{\circ}\text{OH}$ : enhancement or suppression of IgG binding in groups A (common type) and B (rare subset), respectively. The avidities of group A antibodies for fluid-phase  $\beta_2$ GPI were low but increased in a dose-dependent manner upon  $\beta_2$ GPI irradiation, in relation to protein crosslinking. Distinguishing features of group B antibodies included higher avidities for fluid-phase  $\beta_2$ GPI that was no longer recognized after  $^{\circ}\text{OH}$  treatment, and negative anticardiolipin tests suggesting epitope location near the phospholipid binding site. The  $^{\circ}\text{OH}$  scavengers thiourea and mannitol efficiently protected against all above changes. In contrast, the Fenton system induced no major alteration in the structure and functions of  $\beta_2$ GPI.

Thus, oxidative modifications of  $\beta_2$ GPI via  $^{\circ}\text{OH}$  attack of susceptible amino acids alters phospholipid binding, and modulates recognition by autoantibodies depending on their epitope specificities. These findings may be of clinical relevance for the generation and/or reactivity of anti- $\beta_2$ GPI antibodies.

### P83

#### Isolation of $\beta_2$ GPI by perchloric acid yields three proteins having different antigenic properties

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The precipitation by perchloric acid is usually the first step in the isolation of  $\beta_2$ GPI used in the ELISA for anti- $\beta_2$ GPI antibodies. Perchloric acid inhibits plasmin and denaturates most proteins except for those being very basic.

The aim of our study was to evaluate the common procedure for the isolation of  $\beta_2$ GPI with special emphasis on the precipitation step with perchloric acid.

The precipitation by perchloric acid, performed with different timing (3, 18 or 50 minutes) was followed by affinity chromatography on heparin, concluded by cationic exchange chromatography. Elution with the  $\text{Na}^+$  gradient (linear 0.05-0.65M) led to three distinct protein peaks. Each peak was isolated and analysed separately by 1./denaturated polyacrylamide electrophoresis, 2./rocket electrophoresis with rabbit polyclonal anti- $\beta_2$ GPI and 3./ELISA with 6 SLE and/or APS patients' sera, previously determined by the standard anticardiolipin and anti- $\beta_2$ GPI ELISA. In the ELISA all 9 proteins were used at the same concentration, determined by colorimetric reaction.

The protein from the 2<sup>nd</sup> peak exhibited a molecular weight of 50 kDa, corresponding to both molecular weight markers and reference  $\beta_2$ GPI (Tincani, Brescia Italy). Both the protein from the 1<sup>st</sup> and 3<sup>rd</sup> peak exhibited a molecular weight of about 55 kDa. The same result was observed after all three different precipitations. In rocket electrophoresis, the proteins from the 2<sup>nd</sup> and 3<sup>rd</sup> but not from the 1<sup>st</sup> peak reacted with polyclonal rabbit anti- $\beta_2$ GPI antibody. There were no differences in the activities among the isolates obtained by the different precipitation timing. In the ELISA, the proteins from the 2<sup>nd</sup> and 3<sup>rd</sup> but not from the 1<sup>st</sup> peak reacted with human anti- $\beta_2$ GPI antibodies. Differences among the isolates obtained by different precipitation timing were observed. The protein from the 2<sup>nd</sup> peak obtained after 3-minute precipitation reacted 2 to 10 times stronger with different patients' sera than the protein from the 3<sup>rd</sup> peak (from the same isolation). Proteins obtained after 18-minute precipitation reacted more weakly than did the proteins from the isolation after 3-minute precipitation, but the protein from the 2<sup>nd</sup> peak gave still 2 to 6 time higher results than

the protein from the 3<sup>rd</sup> peak. The proteins from the 2<sup>nd</sup> and 3<sup>rd</sup> peaks after 50-minute precipitation reacted almost equally; the protein from the 3<sup>rd</sup> peak after 50-minute precipitation reacted stronger than the same protein either after 18-minute or 3-minute precipitation.

In conclusion, the precipitation with perchloric acid followed by affinity purification on heparin and cation exchange chromatography with linear  $\text{Na}^+$  gradient (0.05 to 0.65M) yielded three different proteins, out of which one was antigenically nonactive and two were antigenically active with anti- $\beta_2$ GPI antibodies. The time of precipitation influenced the antigenic properties of the two proteins with the same antigenic specificity but different molecular weight.

### P84

#### Antibodies to $\beta_2$ -glycoprotein I, prothrombin and antithrombin III as markers of the antiphospholipid syndrome severity

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Antibodies to  $\beta_2$ -glycoprotein I / $\beta_2$ -GP I/, prothrombin /Pt/ and antithrombin III /AT III/ are not strictly speaking antiphospholipid antibodies, but they are closely associated with this pool of antibodies. The "marker" and prognostic significance of these antibodies have been recently widely discussed.

The aim of the presented study was to determine whether the anti- $\beta_2$ -glycoprotein I, anti-Pt and anti-AT III antibodies possess "marker" and prognostic value in APS syndrome.

The presence of the serum antibodies to these serum proteins, six selected phospholipids /including cardiolipin/ and Lupus antycoagulant have been tested and correlated with such clinical manifestations of the antiphospholipid syndrome /APS/ like: thrombotic events, fetal loss, thrombocytopenia and livedo reticularis. The study covers 83 sera of the patients with various diagnoses /in 11 SLE, 43 SLE + APS /SAPS/ and 29 in PAPS/. The antibodies to  $\beta_2$ -glycoprotein I in 21% of the SAPS cases and in 24% of the PAPS cases have been found and in none of the SLE without APS syndrome sera. The antibodies to Pt are present in 20% and to AT III in 15% of the tested sera. The correlation of antibodies to negatively charged phospholipid, LAC and anti- $\beta_2$ -glycoprotein I seems to be evident. Also significant increasing of the frequency of the appearance of selected clinical manifestations in the group of the anti- $\beta_2$ -glycoprotein I, anti-Pt and anti-AT III – positive sera was observed, especially fetal loss, livedo reticularis and thrombocytopenia. It seems to us the anti- $\beta_2$ -glycoprotein I, anti-Pt and anti-AT III antibodies are very promising "marker" for APS and possess also prognostic value.

### P85

#### Differences between active immunoinflammatory and postinfectious fibromyalgia (FM)

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**Aim:** To study immunological and neurochemical markers in cerebrospinal fluid (CSF) and serum of FM patients in two subgroups, one having had a slow onset of symptoms (SO) and the other an acute onset (AO) of FM after a flue-like attack.

**Materials:** Twenty women with SO and 19 with AO FM, matched as to age and clinical symptoms, were studied for a multitude of antimicrobial and autoantibodies in serum. Markers of inflammation, immune activation and nerve cell damage were looked for in CSF and serum. All patients had longstanding disease.

**Results:** More patients with AO FM had IgM antibodies to enteroviruses, but PCR amplification showed no signs of enteroviral genome in CSF. All other antimicrobial and autoantibodies were similar in the two groups. However, in the SO FM patients we found strongly increased intrathecal IgA production as shown by extended indices but normal albumin ratio indicating normal blood/CSF barrier function. Intrathecal IgM production was increased in a few SO FM patients but IgG production was normal in all FM patients. Myelin basic protein (MBP) levels were normal in CSF of AO FM patients but very low in the SO patient group.

**Conclusions and discussion:** In FM characterized by an insidious onset of symptoms an immunoinflammatory mechanism involving IgA production in the brain may be a driving pathogenetic mechanism. Patients having experienced an acute onset of FM after a flue-like episode are likely to suffer from sequelae after earlier encephalitis, showing no signs of immune activation. The abnormally low MBP levels in the CSF of SO FM patients are yet unexplained. Our findings strongly support the concept that FM is a result of brain abnormalities that lead to disordered sensory processing and widespread allodynia.

## P86

### Disorders of the system of hemostasis and biochemical parameters of NZB/NZW F1 mice

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Object of a research. A research of interaction of coagulation and biochemical parameters of NZB/NZW F1 mice with spontaneous explicating lupus like disease.

**Methods:** 120 female mice of a line NZB/NZW F1 3 months age were investigated. Coagulation tests were used: counting platelets, activated partial thromboplastin time (APTT), thrombin time (TT), prothrombin time (PT), concentration of fibrinogen, soluble fibrin monomer complexes (SFMC); parameters of platelet aggregate (spontaneous and induced with ristocetin, collagen and ADF). Biochemical parameters of serotonin and cortisol were investigated. An electronic microscopy of microvessels was investigated also.

**Results:** Significant (more than twice) decreasing the amount of platelets of NZB/NZW F1 mice, elongation of parameters of the coagulation tests (APTT  $40,0 \pm 2,7$  sec, control  $27,6 \pm 2,5$  sec) ( $P < 0,01$ ), decreasing the concentration of fibrinogen ( $1,1 \pm 0,2$  g/l, control  $5,2 \pm 0,6$  g/l), increasing the level SFMC ( $28,1 \times 10^{-2} \pm 1,6$  g/l, control  $8,9 \times 10^{-2} \pm 1,03$  g/l), increasing the parameter of spontaneous platelets aggregate ( $20,3 \pm 1,96$  %, control  $2,5 \pm 0,6$  %) and aggregate of platelets with ADF ( $12,8 \pm 1,3$  %, control  $9,0 \pm 0,8$  %) decreasing the aggregate with collagen ( $4,4 \pm 0,6$  %, control  $9,3 \pm 0,8$  %) were registered. The concentration of "plasma" serotonin was increased ( $0,065$  mcg/ml, control  $0,042$  mcg/ml), the level of cortisol was considerably reduced ( $0,4 \pm 0,09$  mcg/ml, control  $1,03$  mcg/ml). The correlation between increasing the concentration of "plasma" serotonin, increasing the parameter of the spontaneous aggregate of platelets, increasing the concentration of SFMC, elongation of the coagulation tests and decreasing the concentration of "platelet" serotonin were marked. By the electronic microscopy the dystrophy of endothelium is registered.

**Conclusion:** Thus the endothelial damage of NZB/NZW F1 mice was accompanied by the expressed activation of a system of hemostasis, amplifying the aggregate of platelets and increasing the

release of serotonin from them. At the same time the significant decreasing the concentration of cortisol was found. These disorders of hemostasis are typical for DIC syndrome. Therefore, it is possible to use NZB/NZW F1 mice as an animal model for study of disorders of hemostasis, including DIC syndrome, for the patients with SLE.

## Poster Discussion F

### Innovative Therapies

## P87

### Adenoviral gene transfer of tissue inhibitors of metalloproteinases (TIMPs) reduces the invasive behaviour of rheumatoid fibroblast-like synoviocytes

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In rheumatoid arthritis (RA), an excess of proteolytic enzymes secreted at the synovium-cartilage junction results in the invasion of the articular cartilage by synovial cells. The aims of the present study were to investigate the effects of overexpression of TIMP-1 and TIMP-3 on: 1) the invasive behaviour of rheumatoid fibroblast-like synoviocytes and 2) cell proliferation and apoptosis.

The day before the experiments, the synoviocytes were infected with adenoviral vectors encoding TIMP-1 or TIMP-3 or with a control vector (AdLacZ). A Transwell system was used to study invasion of the cells. After 3 days, the invaded cells were counted using a microscope. Proliferation was assessed by measuring 3H-thymidine incorporation and cell counting. Apoptosis was assessed at 1-4 days after transduction using an Annexin V-FITC kit.

Both TIMP-1 and TIMP-3 overexpression resulted in a significant reduction, respectively 60% ( $P < 0.001$ ) and 80% ( $P < 0.001$ ), of invasiveness of the synoviocytes as compared to the AdLacZ-transduced cells. In all cases, TIMP-3 was superior to TIMP-1 ( $P = 0.02$ ). Cell proliferation was significantly reduced by TIMP-3 overexpression (40%;  $P < 0.05$ ) and to a lesser extent by TIMP-1 (20%;  $P < 0.05$ ) as compared to AdLacZ. There were little differences in % of apoptotic cells between the non-transduced, AdTIMP-1, AdTIMP-3 or Ad LacZ transduced cells up to 4 days after transduction. A maximum of 15% of the AdTIMP-3 transduced cells were in apoptosis as compared to a maximum of 12% in the other conditions. These results show that the invasive behavior of RA-FLSs can be strongly inhibited by overexpression of TIMPs. Both MMP inhibition and a reduction of proliferation appear to contribute to this effect. The superior effect of TIMP-3 may be due to a stronger effect on proliferation or to differences in the inhibitory profile of TIMP-1 and TIMP-3. To limit joint destruction in rheumatoid arthritis, inhibition of cartilage invasion by the pannus tissue by TIMP overexpression may be a useful approach.

P88

### Adenoviral-based overexpression of TIMP-1 reduces tissue damage in the joints TNF-transgenic mice

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**Introduction:** Rheumatoid arthritis is a prototype of a destructive inflammatory process. Inflammation triggered by the overexpression of TNF- $\alpha$  is recognized as a driving force of the disease process and mediated tissue destruction. The particular impact of TNF- $\alpha$ -dependent pathways in tissue destruction is unknown.

**Materials and methods:** Herein, the effect of an overexpression of tissue inhibitor of metalloproteinases (TIMP)-1, a physiological antagonist of metalloproteinases, was studied in the arthritis model of TNF- $\alpha$ -transgenic mice. Systemic treatment was carried out by replication defective adenoviral vectors for TIMP-1 (AdvTIMP1,  $n = 7$ ) or  $\beta$ -galactosidase (AdvLacZ,  $n = 6$ ) or phosphate buffered saline (PBS,  $n = 7$ ), which were injected once intravenously at the onset of arthritis. Clinical, serological, radiological and histological outcomes were assessed 18 days after treatment.

**Results:** The AdvTIMP1 group showed a significantly improved clinical outcome as measured by paw swelling and grip strength than the two control groups, whereas total body weight, TNF- $\alpha$  and IL-6 levels were similar in all groups. Tissue destruction as assessed by X-ray and histology of hind paws was significantly lower in the AdvTIMP1 group than in the AdvLacZ- and PBS- control groups. Finally, the formation of arthritis-specific autoantibodies to hnRNP-A2 was not observed in the AdvTIMP1 group but were present in the two control groups.

**Discussion:** These results indicate a central role of metalloproteinases in TNF- $\alpha$ -mediated tissue damage *in vivo* and a promising therapeutic role of TIMP-1.

P89

### Serum levels of matrixmetalloproteinases MMP1 (collagenase) and MMP3 (stromelysin) before and after treatment with leflunamide in patients with rheumatoid arthritis

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**Objective:** Leflunamide has been proven to be efficient in reducing joint inflammation and destruction in patients with rheumatoid arthritis (RA). This study was conducted to examine effects of leflunamide on serum levels of matrixmetalloproteinases MMP1 and MMP3 in patients with RA.

**Methods:** In a prospective clinical trial, we measured in 24 patients suffering from RA, as defined by ACR criteria, serum activities of MMP1 and MMP3 by means of ELISA. Analysis of MMPs was performed before and after a treatment period of approximately 3 months ( $84 \pm 14$  days, mean  $\pm$  SD) with leflunamide. Additionally, conventional inflammatory parameters (CRP, ESR) and clinical data of RA activity were determined.

**Results:** Leflunamide treatment led to a highly significant reduction of MMP1 serum activity ( $p < 0.001$ ), whereas MMP3 values were not influenced. Furthermore, the number of painful ( $p < 0.01$ ) and swollen ( $p < 0.05$ ) joints decreased significantly as well as clinical inflammatory joint activity scores (GLASS,  $p < 0.001$ ) and levels of CRP ( $p < 0.05$ ).

**Conclusion:** In accordance with recent data, leflunamide is effective in reducing the clinical inflammatory activity of RA, and in decreasing the activity of matrix-degrading factors like MMP1. The differential effect of this immunomodulative drug on MMP1 and MMP3 will be explored in further investigations.

P90

### Altered migratory capacity of polymorphonuclear leucocytes as an effect of TNF-alpha blockade in patients with rheumatoid arthritis

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Rheumatoid Arthritis (RA) is associated with progressive joint destruction, functional disability and decreased life expectancy. Although the underlying cause of RA is unknown, TNF- $\alpha$ , a proinflammatory cytokine, contributes to the pathogenesis of synovitis and joint destruction. Anti-TNF-biological response modifiers, such as Etanercept a recombinant human TNF receptor fusion protein, suggest that TNF- $\alpha$ -inhibition is a viable approach to control disease activity in RA.

Considering the pivotal role TNF  $\alpha$  plays in the first line defense against bacterial and fungal infections by polymorphonuclear leucocytes (PMN) it seems important to focus on the PMN function in RA patients, treated with Etanercept. Since a 29% increase in infections of the upper respiratory tract under TNF- $\alpha$  blockade has been reported, we investigated inflammatory parameters related to PMN-activity in 6 RA-patients, before and after 3-months of Etanercept treatment without altering their methotrexate and/or glucocorticoid-medication. The migration of the neutrophils was measured with a standardized whole blood membrane filter assay with and without stimulation by the chemoattractant fMLP. The percentage of migrating PMN (total migration index, TMI) and the relative penetration depth into the filters (distribution characteristics, DC) served to characterize the migratory behaviour of neutrophils. To estimate *in vivo* granulocyte activation, neutrophil elastase was measured in plasma from RA patients. In addition, PMN-blood count and C-reactive protein as a marker of disease activity, were analysed. TNF- $\alpha$  blockade significantly ( $p < 0,03$ ) reduced the spontaneous and fMLP stimulated median TMI (15.2 vs. 10.9 and 16.5 vs. 11.4, respectively). Moreover the spontaneous and the fMLP-stimulated median DC was reduced under Etanercept-treatment ( 21.1 vs. 5.9,  $P < 0.03$  and 20.5 vs. 6.6,  $P < 0.06$  respectively). Interestingly, these values were still within the normal range of migratory activity. Furthermore, TNF blockade significantly reduced the median plasma elastase levels ( 252 vs. 108,  $p < 0,04$ ), as well as the median C reactive protein levels ( 21 vs. 7  $P < 0,035$ ) and the median number of polymorphonuclear leucocytes ( 7.73 vs. 4.58,  $P < 0.0001$ ). Of note, plasma elastase levels as a sign of systemic PMN-activation were still above the normal range. During the observed treatment period all patients showed an improvement in the inflammatory symptoms of RA ( 2 had a 70% ACR-response, 3 a 50% and 1 a 20% ACR response). No patients had signs of bacterial or fungal infections. In conclusion, TNF  $\alpha$  blockade did not suppress PMN migratory activities to levels that are associated with higher incidences of infections.

## P91

### Development of a doxycycline inducible AAV vector for long term *in vivo* viral IL-10 gene transfer in rheumatoid arthritis

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**Objectives.** The recent development of AAV vectors (adeno-associated virus) offers new perspectives for cytokine gene transfer in RA as they are non pathogenic and allow long term transgene expression *in vivo*. Moreover, we propose to regulate vIL-10 expression with tetracycline derivative (tetON system). The purpose of this study was to assess the potential long-term gene expression regulation of a recombinant AAV vector expressing vIL-10 in human rheumatoid synovial tissue and its efficiency in collagen-induced arthritis (CIA).

**Methods.** The AAV-tetON-vIL10 vector contains two transcriptional units oriented in opposite directions, with a central bi-directional SV40 polyA. Sequences encoding the transcriptional activator rTA, which confers doxycycline transgene induction, is inserted downstream a retroviral LTR promoter. A minimal human CMV promoter, flanked with tetracycline operator motifs, controls the transcription of vIL-10. Human RA synoviocytes were infected *in vitro* with AAV-tetON-vIL10 (500 MOI) and vIL-10 secretion was assessed by ELISA after addition of 5 µg/ml doxycycline (dox). Therapeutic efficiency of the vector was achieved after intra-muscular injection ( $1.5 \times 10^9$  pi) in DBA1 mice with CIA in the presence of doxycycline in the drinking water (0.2 mg/ml).

**Results.** Viral IL-10 secretion by RA synoviocytes was increased 40-fold in presence of dox (233 ng/ml/ $10^6$  cells) and returned to basal level 24 hr after dox removal. In CIA, serum vIL-10 increased to 0.38 ng/ml, 5 weeks after gene transfer in animals under diet dox. RT-PCR analysis showed vIL-10 transcription in the muscle up to 14 weeks, without diffusion in other organs. We observed a decrease of CIA incidence (30% versus 89% in AAV-GFP injected control group) and of paw swelling ( $1.68 \pm 0.04$  versus  $1.81 \pm 0.15$  on day 35 post-immunization,  $P < 0.0003$ ).

**Conclusions.** AAV vectors conferred safe and inducible long-term expression of vIL-10. These data support AAV-tetON-vIL10 as a therapeutical tool for gene therapy in RA.

## P92

### IL-18 blockade is a potential disease-modifying therapy for rheumatoid arthritis

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**Introduction:** Interleukin-18 (IL-18) has been demonstrated as promoting the development of a TH1 response *in vivo* in synergy with IL-12. Significant levels of IL-18 and IL-12 have been detected in the joints of patients with rheumatoid arthritis (RA).

**Aim:** To define the therapeutic potentials of IL-18 blockade in RA by investigating the effect of neutralising endogenous IL-18 in the experimental CIA mouse model.

**Methods:** Two distinct IL-18 neutralising strategies, i.e., a recombinant human IL-18 binding protein (hIL-18BP) and a polyclonal anti-IL-

18 IgG, were used to treat CIA mice in a therapeutic protocol (after disease onset). The effect on disease severity (visual scores) as well as parameters of cartilage and bone destruction were evaluated.

**Results:** Clinical scores were significantly reduced after IL-18 blockade (rhIL-18BP 1 mg/kg,  $P < 0.001$ ,  $n = 13$ ; rhIL-18BP 0.25 mg/kg,  $P < 0.05$ ,  $n = 7$ ; anti-IL18 IgG, 2 mg,  $P < 0.05$ ,  $n = 9$ , Mann Whitney test, treated versus placebo groups). Histological examination showed cartilage protection (decrease erosion scores,  $P < 0.05$ ) that was accompanied by significantly reduced levels of serum cartilage oligomeric matrix protein (an indicator of cartilage turnover) and VDIPEN expression (a neoepitope present after digestion by matrix metalloproteinases). X-ray analysis of joints provided evidence of reduced bone erosion. Serum IL-6 levels were diminished in the treated animals.

**Conclusions:** These results clearly demonstrate that blocking endogenous IL-18 is therapeutically efficacious in the CIA model and support the use of IL-18 neutralisation as a novel cartilage and bone protective therapy for the treatment of destructive arthritis. Recombinant hIL-18BP could therefore represent a new disease-modifying anti-rheumatic drug that warrants testing in clinical trials in patients with rheumatoid arthritis.

## P93

### Digital vasculitis in a patient with rheumatoid arthritis: good response on anti-TNF blockade

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Rheumatoid arthritis (RA) may be complicated by vasculitis. Vasculitis usually affects small vessels of the skin causing nailfold infarcts, but may also affect larger vessels and cause severe damage to internal organs. In such cases, treatment with high doses of corticosteroids or other immunosuppressive drugs may be necessary. TNF-alpha blockade has been shown to be an effective and safe treatment for RA, but thus far no reports have addressed the effect of TNF-alpha blockade on extra-articular manifestations of RA, such as vasculitis. We report a patient with RA and nailfold infarcts which repeatedly disappeared for several weeks following monthly i.v. injections with an anti-TNF alpha receptor fusion protein.

A 46 year old woman was diagnosed as having rheumatoid factor positive, erosive RA in 1982. Due to the uncontrollable disease she was included in 1994 in a study with Ro 45-2081, a fusion protein combining two p55 TNF receptors with the Fc component of an IgG human antibody (Roche, Basel, Switzerland, sTNFR:Fc). After a three months placebo controlled phase she was treated with 50mg sTNFR:Fc every four weeks. Clinical response was impressive with swollen joint counts decreasing from 32 to 5 and C-reactive protein CRP levels declining from 95 at baseline to 20 after the first injection. Low disease activity was sustained for the following years. Besides sTNFR:Fc her medication consisted of oral prednisone 5 mg a day and occasionally paracetamol 500 mg. In the spring of 1999 she first noticed nailfold infarcts on the fingers of both hands. These lesions disappeared after every injection of sTNFR:Fc and reappeared three weeks thereafter when the clinical effects of sTNFR:Fc were decreasing. This effect on the digital vasculitis has been well documented during several cycles of sTNFR:Fc administration.

**Conclusion:** The prompt disappearance of nailfold infarcts after sTNFR:Fc administration observed in our patient strongly suggests a therapeutic effect of sTNFR:Fc on active vasculitis. This observation raises the question whether blocking of TNF-alpha might also be effective in more severe forms of vasculitis and possibly other extra-articular manifestations of RA, some of which are life threatening and are currently treated with high doses of corticosteroids and immunosuppressive drugs.



**P94****Effect of osteoprotegerin and pamidronate treatment in transgenic mice overexpressing human TNF****K Redlich\***, **A Maier†**, **S Hayer\***, **G Kollias‡**, **CR Dunstan§**, **M Tohidast-Akrad\***, **S Lang#**, **W Woloszczuk\*\***, **G Steiner\***, **JS Smolen\*** and **G Schett\***

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Rheumatoid arthritis (RA) is characterized by progressive joint destruction resulting from chronic inflammation. Recent studies suggest that bone-resorbing osteoclasts formed in the synovium play an important role in bone destruction in RA. We studied the effect of anti-resorptive treatment with osteoprotegerin and/or pamidronate compared to TNF blockade with infliximab on the development of erosions in TNF overexpressing mice. Systemic treatment with osteoprotegerin (OPG), pamidronate, both osteoprotegerin and pamidronate, infliximab or phosphate buffered saline (PBS) was carried out by intravenous injection. Treatment was initiated at the time of onset of arthritis and continued over 35d. Clinical, serological, radiological and histological outcomes were assessed after treatment. Clinical improvement, as assessed by reduction in paw swelling was only seen in the infliximab treated group. X-Rays of the hind paws were performed to quantify erosive changes. Erosions were detectable in each joint compartment. Grading of erosions was performed analogous to the Larsen score. There was a marked and significant ( $P < 0.05$ ) reduction in the Larsen scores of mice treated with OPG (-54%), OPG and pamidronate (-64%) and infliximab (-66%). Microscopic examination of decalcified joint tissue sections using a semiquantitative method, revealed a significant ( $P < 0.05$ ) reduction in the extent of erosions in all treatment groups (OPG: -56%; pamidronate: -53%; OPG + pamidronate: -81%; infliximab -46%) when compared to controls. These data suggest that anti-resorptive treatment may have a significant potential in TNF-mediated bone destruction.

**P95****Induction of a rapid progressive cartilage destruction in SCID mice by intraarticular application of a murine fibroblast like cell line****U Sack**, **A Hirth**, **B Funke**, **K Wiedemeyer**, **S Konrad**, **J Lehmann** and **F Emmrich**

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**Background:** In pathogenesis of rheumatoid arthritis, fibroblasts are considered to be a crucial cell population for disease progression as well as joint destruction. Following intraarticular injection into SCID mice, isolated human rheumatoid synovial fibroblasts have been shown to induce a destructive arthritis (hu/mu SCID arthritis). Although exclusively human synovial fibroblasts were able to induce this arthritis, cartilage destruction was caused by murine fibroblast like cells in this model. Therefore, we have isolated a murine destructive fibroblastoid cell line and established a cartilage destruction model.

**Material and methods:** LS48 cell line was examined for morphological, ultrastructural, immunological, and functional parameters. Furthermore, cartilage destruction was induced by intraarticular application of LS48 cells into SCID mouse knee joints. Mice were monitored for joint swelling, serological parameters and by radiolog-

ical methods. Finally, immunohistochemistry and histology were used to characterize morphology of cartilage destruction.

**Results:** LS48 was shown to present characteristics of a fibroblast-like cell. Secretion of interleukin-6 and tumor necrosis factor-alpha revealed similarities to human invasive rheumatoid synovial membrane fibroblasts. Installation of  $5 \times 10^5$  cells into SCID mouse knee joints caused a rapid progressive process causing cartilage destruction within 10 days. Morphology revealed infiltration of fibroblast like cells into the cartilage.

**Conclusions:** Induction of cartilage destruction by intraarticular application of these murine fibroblast like cells is a rapid and highly reproducible model for investigation of cartilage destruction in arthritic joints. This provides an excellent possibility to investigate relevant processes and new therapeutic strategies for rheumatoid arthritis in an easy-to-handle animal model.

**P96****Infiltrate analysis of rheumatoid synovial tissue before and after high dose chemotherapy and autologous stem cell transplantation****RJ Verburg**, **R Flierman**, **EWN Levahrt**, **F van den Hoogen\***, **FC Breedveld** and **JM van Laar**

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**Objective:** To investigate the effects of high dose chemotherapy (HDC) and autologous stem cell transplantation (ASCT) on the synovial infiltrate in rheumatoid arthritis.

**Methods:** 8 patients with erosive, refractory, progressively rheumatoid arthritis, were treated with HDC (cyclophosphamide 200 mg/kg) and CD34 enriched selected ASCT. Biopsies of synovial tissue from a clinically involved knee were obtained by arthroscopy before and three months after HDC and ASCT. Immunohistochemistry was performed and blindly scored on a five point scale (0-4) using MoAbs specific for the following markers: CD3, CD4, CD8, CD25, CD27, CD45RA, CD45RO, CD45RB, CD19, CD20, CD22, CD38, CD5, CD68, HLA-DR, CD62L, CD62E, CD56 and CD55.

**Results:** There were no statistical significant differences (Wilcoxon's signed rank test) when the results before and after transplantation were compared. However when patients were divided in clinical responders (ACR > 50%,  $n = 5$ ) and non-responders (ACR < 20%,  $n = 3$ ) statistically significant differences with respect to several T-cell markers were found (Table).

|        | Responders |           | Non-responders |           | <i>P</i> * |
|--------|------------|-----------|----------------|-----------|------------|
|        | Before     | After     | Before         | After     |            |
| CD 3   | 2.8 ± 0.5  | 1.3 ± 2.3 | 1.0 ± 1.4      | 1.7 ± 2.1 | 0.06       |
| CD27   | 3.0 ± 0.8  | 0.3 ± 0.6 | 1.0 ± 0.8      | 1.7 ± 2.1 | 0.05       |
| CD45RA | 2.3 ± 0.9  | 0.7 ± 1.2 | 0.5 ± 1        | 1.6 ± 2.1 | 0.03       |
| CD45RO | 3.4 ± 0.5  | 0.7 ± 1.2 | 1.8 ± 1.6      | 2.0 ± 2   | 0.04       |
| CD45RB | 3.2 ± 0.8  | 1.0 ± 1.7 | 2.0 ± 1.6      | 2.0 ± 2.0 | 0.05       |

Mean Histological Score ± STDEV. \* Mann-Whitney U test. Clinical and immunohistochemical responses were predicted by CD27 ( $P = 0.016$ ), CD45RO ( $P = 0.003$ ) and CD45RB ( $P = 0.047$ ) infiltration before treatment.

**Conclusions:** There was a statistically significant difference ( $P < 0.05$ ) between clinical responders and non-responders with respect to a decrease in infiltration of CD45RA+ and CD45RO+ cells after HDC and ASCT. CD27, CD45RO and CD45RB appear to be useful markers to predict clinical response.

## P97

### Newer immunomodulating drugs in rheumatoid arthritis may precipitate glomerulonephritis

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Three patients with rheumatoid arthritis on newer immunomodulating therapy, developed acute glomerulonephritis. Two of the patients were treated with tumour necrosis factor blockade ( Etanercept, 25 mg sc. twice a week) and one with leflunomide (Arava, 20mg daily) in addition to the conventional medical treatment. All of the patients developed unexpected blood and urinary abnormalities, two of them after treatment with Etanercept for eleven- and one month respectively. Renal biopsies showed in the patients with long term Etanercept treatment, focal proliferative glomerulonephritis with cellular crescents in 30% of all glomerular sections. The biopsy showed mesangial deposits of IgA. This patient was suspected clinically for subacute bacterial endocarditis, however all data were negative. In the other patient treated with Etanercept for only four weeks slightly diffuse mesangial proliferative glomerulonephritis was demonstrated. Electron microscopy of this biopsy showed distinct mesangial matrix changes "moth-eaten" appearance. In the patient treated with Arava during four weeks, biopsy showed focal proliferative glomerulonephritis with cellular crescents in 7% of all glomerular sections and with IgA mesangial deposits.

Two of the patients thus had IgA glomerulonephritis. The diagnosis of the third one was inconclusive, as regard the present of IgA, but pathology could represent IgA glomerulonephritis in resolution.

The relation in time of sign of renal disease to the treatment with Etanercept and Arava makes it probable that renal disease was related to these drugs. It is generally assumed that IgA glomerulonephritis is caused by the deposition of immune complexes, but details of antigen(s) are in these cases unknown. We finally regard it as a possibility that the immunomodulation caused by these new drugs may facilitate silent infection and subsequently development of IgA glomerulonephritis. At least in long term treated patients this aetiology could not be excluded.

## P98

### The use of Ribomunyl® in the immunomodulatory treatment of rats with adjuvant arthritis

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Immunomodulatory therapy of inflammatory rheumatic diseases, especially in refractory forms of systemic lupus erythematosus (Rovensky *et al.*) and rheumatoid arthritis (Mateicka *et al.*) has its tradition. The very immunosuppressive therapy may induce the development of resistance and to participate in recurrent secondary infections in patients suffering from systemic diseases of the connective tissue. Alternatives of immunomodulatory therapy are therefore sought for that would eliminate some adverse effects of immunosuppressive agents on the cell-mediated and non-specific immunity function, and would favorably affect the clinical condition of the patient.

To verify our working hypothesis concerning the appropriateness of immunomodulatory therapy with Ribomunyl®, the adjuvant arthritis model in rats was chosen. Following drugs and their combinations were orally administered to animals in a long-term prophylactic course: Cyclosporine A (CyA, 2,5 mg/kg/day), methotrexate (MTX, 0,3 mg/kg, 2 times a week), Ribomunyl® (25 mg/kg 4 times a

week), CyA+MTX, CyA+Ribomunyl®, MTX+Ribomunyl®, and the three-combination of CyA+MTX+ Ribomunyl®. When given in combination, both the doses and the frequency of administration were the same as when the drugs were administered alone. The following markers of inflammation and arthritic process were measured: serum albumin, joint X-ray, hind paw swelling, and on day 40 of the study, bone mineral density (BMD) and bone mineral content (BMC).

Our results showed that Ribomunyl® alone has no marked effect on markers of inflammation and arthritis in animals with adjuvant arthritis. When combined with the immunosuppressive drugs CyA and MTX, a similar and/or better therapeutical effect was observed than with the basic drug without Ribomunyl®. However, the effect of the three-combination of CyA+MTX+Ribomunyl® was rather remarkable. This combination had the most pronounced therapeutical effect on rats with adjuvant arthritis. It significantly inhibited inflammatory and arthritic markers as well as BMD and BMC reductions.

Our results obtained using the adjuvant arthritis model suggest that immunomodulatory procedures are promising. These results therefore need to be verified in additional animal models and markers of cell-mediated immunity and/or cytokines involved in the induction of this therapeutic effect should be investigated.

Rovensky J. *et al.*: Levamisole treatment of systemic lupus erythematosus. *Arthritis Rheum* 1982; 24: 470-471.

Mateicka *et al.*: Immunomodulatory treatment with Biostim (Roussel Uclaf) in patients with rheumatoid arthritis (Preliminary follow-up of group with 10 patients). *Rheumatologia* 1992;6: 129-133.

## P99

### Thymosin beta4 sulphoxide: potential role in resolution of inflammation?

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**Background:** Thymosin beta 4 sulphoxide (Tb4so) has previously been shown to be produced by glucocorticoid-treated monocytes (1). This highly conserved intracellular peptide possesses 'moonlighting' functions in the modulation of inflammatory responses, and may represent a natural down-regulator of inflammation *in vivo*. We have investigated the mechanisms of action of Tb4so primarily on neutrophils by studying its effects on *in vitro* and *in vivo* models.

**Methods:** Effect of Tb4so on assays of neutrophil function included chemotaxis and respiratory burst. Apoptosis was measured as Annexin-V/PI binding by FACS and macrophages were stained for phagocytic uptake of apoptotic neutrophils by the presence of neutrophil-specific myeloperoxidase. *In vivo*, the effect of administration of Tb4so on carrageenan-induced inflammation was explored.

**Results:** Tb4so significantly inhibited fMLP-induced chemotaxis ( $P < 0.005$ ) and respiratory burst of human neutrophils in a dose dependent manner (100% vs 23%,  $P < 0.05$ ). Further, it increased the rate of apoptosis in neutrophils ( $20.5 \pm 1.9\%$ ,  $P < 0.05$ ) and their subsequent phagocytic uptake by macrophages. *In vivo*, Tb4so was a potent inhibitor of neutrophil mediated carrageenan-induced inflammation in BALB/c mice (1.2mm vs 0.6mm  $P < 0.001$  at 24h).

**Conclusions:** Tb4so is an anti-inflammatory peptide that down-regulates neutrophil mediated inflammation. The mechanism of action appears to be, at least in part, via induction of neutrophil apoptosis and their clearance by phagocytic macrophages. These results suggest therapeutic potential for Tb4so.

**P100****New antirheumatic effects of bisphosphonate treatment****L Konenkova, E Zonova, A Sizikov, M Korolev, S Tsareva and V Kozlov***Institute of Clinical Immunology SB RAMS, Novosibirsk, Russia*

Recently, secondary osteoporosis therapy has been as new strategy for combination treatment rheumatic disease. We have studied the efficacy of pamidronate treatment in the context of combination therapy rheumatic disease.

The complex check up of 27 patients with rheumatic disease: (rheumatoid arthritis (RA) - 15, systemic lupus erythematosus (SLE) - 3, ankylosing spondylitis (AS) - 2, Reiter syndrome (RS) - 2, systemic sclerosis (SS) - 1), average age 46,2 year.

In all of them the decrease of bone mineral density were revealed. Patients had standard non-change cytotoxic therapy, were treated with «Aredia» (pamidronate, Novartis pharma, 30 mg i.v. infusion). The control clinical and laboratory examination were carry out after 3 and 6 month. In 24 (88,9%) patients significant improvement was observed: decrease the tender-joint count and swollen-joint count of 25 percent; erythrocyte sedimentation rate decreased by 11,5 mm per hour; serum level of antiphospholipid antibodies decreased ( $P < 0,05$ ), Ig G serum levels and of circulating erithroid precursors decreased ( $P < 0,05$ ); CD 3+, CD 4+, CD 8+, CD 20+ levels were normalised. The regulary pamidronate infusion may benefit from optimisation of the antirheumatic therapy for patients with secondary osteoporosis.

**P101****Follow-up study of the effect of a three-month course of ciprofloxacin on the late prognosis of reactive arthritis****T Yli-Kerttula\*, R Luukkainen†, U Yli-Kerttula‡, T Möttönen\*, M Hakola§, M Korpela‡, M Sanila†, A Toivanen\****Department of Medicine, \*Turku University, †Satalinna Hospital, ‡Tampere University Hospital and §Jyväskylä Central Hospital, Finland*

**Methods:** In a randomised, double blind, placebo controlled trial, between 1992 and 1996, 71 patients with acute reactive arthritis (ReA) triggered by a gastrointestinal or an urogenital infection were randomly assigned to receive ciprofloxacin or placebo twice daily for three months. There were no statistically significant differences in any variables during the 12-month follow-up. The aim of the present study was to evaluate the effect of ciprofloxacin on the late prognosis of ReA. We reviewed the long-term outcome in 56 (79%) of 71 patients 4-8 years after the acute phase of ReA. The patients suspected to have inflammatory back pain (IBP) were further evaluated using the MRI of the sacroiliac joints. Six of the 10 patients with chronic spondyloarthritis (SpA) were assessed by Tc-labelled leucocyte scintigraphy to search for the association between gut inflammation and SpA.

**Results:** Two patients (7%) in the active treatment group and 10 patients (36%) in the placebo group had developed chronic disease. Ankylosing spondylitis (AS) was diagnosed in 3 patients (11%) in the placebo group. One patient (4%) in the active treatment group had psoriatic arthritis. None in the active, 4 (14%) in the placebo group were assessed as having undifferentiated spondyloarthritis (USpA, ESSG, Amor's criteria). Three of these USpA patients had sacroiliitis, 1 had chronic oligoarthritis and one male patient had chronic oligoarthritis and bilateral sacroiliitis. Two patients (7%) in the placebo group suffered from chronic enthesiopathy (achilles tendon). Seronegative rheumatoid arthritis was diagnosed in one female patient in the active treatment group. One

patient (4%) in the placebo group had recurrent anterior uveitis. MRI indicated sacroiliitis in 3 out of 5 patients with suspected IBP (2 bilateral, 1 unilateral). Scintigraphy revealed mild (grade 1/4) bowel inflammation in only 2 patients out of 6 with chronic SpA.

**Discussion:** The result is somewhat surprising considering that antibiotics did not show any beneficial effect in the first phase of the study. In this follow-up the occurrence of chronic development or late sequelae was clearly more prominent in the placebo group.

**P102****Early treatment of recent-onset rheumatoid arthritis patients impairs the effect of HLA class II antigens on the progression of joint destruction****LR Lard\*, JMW Hazes\*, FC Breedveld\*, GMTH Schreuder†, RRP de Vries‡, E Zanelli† and TWJ Huizinga\****Departments of \*Rheumatology and †Immunohematology and Blood Transfusion, Leiden University Medical Center, the Netherlands*

**Introduction:** HLA class II antigens influence disease progression as measured by extend of joint destruction in RA. This effect is supposed to be caused by formation of autoreactive T-cells after presentation of (auto)antigens in the context of HLA class II. To investigate whether in patients with recent-onset RA early DMARD treatment could prevent the involvement of autoreactive T cells, we analyzed the association of HLA class II and joint damage in 110 patients with early RA that were treated according to the pyramid strategy with DMARDs and 98 patients with early RA that were promptly treated with DMARDs at two weeks after the first visit.

**Methods:** DNA isolation, DRB1 typing and subtyping and DQB1 typing were performed. Extend of joint damage was measured by the modified Sharp score of the radiographs of hand and feet.

**Results:** In the early treatment group the increase in the median Sharp score in the SE+ group was 1.0 to 5.0 in contrast to 1.0 to 3.0 in the SE- group ( $P > 0.5$ ). However, in the pyramid group the increase in joint damage was 0.0 to 16.0 in the SE+ group in contrast to 0.0 to 5.0 in the SE- group ( $P < 0.005$ ). On the other hand, in the RF+ group of the early treatment group the increase in joint damage was 1.0 to 6.5 in contrast to 1.0 to 1.0 in the RF- group ( $P < 0.005$ ). In the pyramid group the increase in joint damage was 0.5 to 17.0 in the RF+ in contrast to 0.0 to 3.0 in the RF- group ( $P < 0.005$ ).

**Conclusion:** This study shows that early anti-rheumatic drug treatment abolishes the effect of HLA class II alleles on extend of joint damage in early RA patients.

**G. Genetics, apoptosis and miscellaneous****P103****The shared epitope revisited: shared epitope negative HLA-DR alleles influence susceptibility to rheumatoid arthritis****D Reviron, A Perdriger, E Toussiro, D Wendling, N Balandraud, S Guis, G Semana, P Tiberghien, P Mercier and J Roudier***INSERM EMI9940, Rheumatology Ward La Conception and EFS Histocompatibility, Marseille, France; Rheumatology Ward and EFS Histocompatibility Rennes, France; Rheumatology Ward and EFS Histocompatibility, Besançon; France*

We propose to classify HLA-DRB1 alleles into 3 groups: shared epitope positive (SE), shared epitope negative with a positively charged HLA-DR P4 pocket (XP4p) and shared epitope negative with a negative or neutral HLA-DR XP4 pocket (XP4n). Using this classification to analyse 3 different French populations, we find that:

-SE/SE or SE/XP4p genotypes are associated with a high risk to develop RA.  
 -XP4p/XP4p or SE/XP4n genotypes are neutral  
 -XP4p/XP4p or XP4n/XP4n genotypes are protective with respect to the development of RA.

**P104**

**Positivity of HLA-DRB1 rheumatoid epitope does not predict the course of juvenile idiopathic arthritis in Czech children**

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**Aims:** Association of juvenile idiopathic arthritis (JIA) with HLA class II still remains unresolved. Our study investigated whether presence of the HLA-DRB1 amino acid 70-74 rheumatoid epitope (RE) is a predictive factor of the disease course.

**Patients and methods:** We analysed 74 consecutive patients with JIA diagnosed and classified according to ILAR criteria, aged 11.2 ± 4.2 (mean ± SD), 35 boys and 39 girls. The numbers of children having oligoarticular, polyarticular, and systemic form of JIA were 24, 40, and 10, respectively. HLA-DRB1 alleles carrying the QRRAA, QKRAA, and RRRRAA motifs of RE were typed for using PCR with sequence-specific primers.

**Results and Conclusions:** There were no significant differences in frequency of the RE, or its particular motifs, among the three forms of JIA.

|             | JIA oligo. | JIA poly. | JIA systemic | Total | P value |
|-------------|------------|-----------|--------------|-------|---------|
| RE negative | 16         | 25        | 6            | 47    | N.S.    |
| RE positive | 8          | 15        | 4            | 27    | N.S.    |
| Total       | 24         | 40        | 10           | 74    |         |

The 2x2 table were tested using chi2 test with Yate's correction, or Fisher exact test where appropriate.

We therefore conclude that simple positivity of the DRB1 rheumatoid epitope is not a likely predictive factor of the JIA course in Czech children.

**P105**

**In Marseille, Southern France, HLA-B2702 carries higher risk than HLA-B2705 to develop ankylosing spondylitis (AS)**

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HLA-B27 subtypes were defined by molecular typing in 45 patients with AS and 90 controls from Marseille. We subdivided patients and controls in 2 subgroups, according to the birth place of their grandparents: 19 patients and 38 controls constituted the Spanish/North African subgroup; 26 patients and 52 controls constituted the "French" subgroup. In patients from the Spanish/North African subgroup, the frequency of B2702 was higher (74%) than in controls from the same area (21%)*pc*<0.01 and the frequency of B2705 was lower in patients (25%) than in controls (79%)*pc*<0.01. In patients from the French group, the frequency of B2702 was higher (7%) than in controls (2%)(N.S.).

Thus, in the population of Southern France, B2702 seems to carry higher risk to develop AS than B2705.

**P106**

**Juvenile idiopathic arthritis is associated to a functionally active polymorphism in the SH2D2A gene**

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**Objective:** T cell specific adapter protein (TSA<sub>d</sub>) is involved in the negative control of T cell activation. The SH2D2A gene encoding TSA<sub>d</sub> is located on chromosome 1q21 which has been implicated in susceptibility to experimental autoimmune disorders in the mouse (chronic allergic encephalomyelitis and collagen-induced arthritis). Recently we found that short alleles of the SH2D2A gene promoter are associated with multiple sclerosis (MS). The aim of our study was to investigate whether the SH2D2A promoter polymorphism contributes to the genetic susceptibility to develop juvenile idiopathic arthritis (JIA).

**Methods:** DNA from 212 Norwegian patients with juvenile arthritis (categorized as systemic (*n* = 18), poly- RF+ (*n* = 12), poly- RF- (*n* = 61), oligo- (*n* = 87) and extended oligoarthritis (*n* = 31)) and 279 healthy unrelated Norwegian controls were genotyped for a functional GA repeat polymorphism in the promoter region of SH2D2A gene using an ABI automatic sequencing machine (ABI Prism™ XL377)

**Results:** The frequencies of the two shortest alleles GA13 and GA16 were increased among the JIA patients compared to the control; the GA13 significantly so (0.098 vs 0.053, OR=1,97, *P* = 0,0063). When we divided patients into subgroups only in the RF-positive polyarthritis group of patients there was no increases of any of the short alleles. All other subgroups of JIA showed an increased frequency of GA13, however only in the patients with oligoarthritis the increased frequency of GA13 allele reached significance (0.103, *P* = 0.017). When we analyzed the frequency of short alleles in relation to the occurrence of chronic iridocyclitis (CIC) in the group of patients with oligoarthritis (*n* = 14), we found that 57% of these patients carry at least one short allele compared to 46% of the patients without CIC (*n* = 73).

**Conclusion:** Our data indicate that the short alleles of the SH2D2A promoter associated with JIA patients could contribute to the genetic susceptibility of JIA, similar what we have observed in MS. It is possible that the short allele is a marker for particular clinical presentations. This we will investigate in further details.

**P107**

**Analysis of VH and VL mRNA in single synovial and peripheral B/plasma cells of patients with rheumatoid arthritis**

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**Introduction:** Synovial tissue in rheumatoid arthritis displays a complex infiltration of many cell types like T and B lymphocytes, plasma cells, follicular dendritic cells, macrophages etc. Presence of B and plasma cells results in secretion of large amounts of multiple pathologic autoantibodies.

**Aim:** To analyze immunoglobulin VH and VL gene usage on the level of mRNA from a single B or plasma cell isolated from synovium and peripheral blood of patients with RA, in order to determine a clonality and a molecular structure of produced antibodies.

**Materials and methods:** RA synovial tissue obtained during synovectomy was enzymatically digested and single synovial B/plasma cells were sorted using immunofluorescent staining with anti-CD19 or anti-CD138. Peripheral B cells were isolated in the same way without enzyme treatment. cDNA library from each single B and plasma cell was generated. Two stage polymerase chain reaction to analyse VH and VL genes was performed. VH, DH and JH or VL and JL gene segments were assigned and somatic mutations determined by comparison with germline sequences on the V BASE/Genbank data base. As a control peripheral B lymphocytes from healthy donors were screened.

**Results:** Analysis of RA synovial mRNA transcripts revealed prevalence of C gamma recombinants that contained rearranged VH1, VH3, VH4, VH5 and VH6 genes. Utilization of VH segments was similar between RA patients and normal subjects, but the accumulation of somatic mutations was elevated in RA synovial and peripheral B cells. We also found preferential utilization of a limited number of VH and DH gene segments. There was increased frequency of kappa light chains containing unusually long CDR3 when compared to normal peripheral B cells.

**Conclusions:** Our findings are consistent with hypothesis that B cell response RA synovium is probably antigen driven and oligoclonal. The project was supported by grant VS961 29 from Ministry of Education, Youth and Sport in the Czech Republic.

## P108

### Disparate associations of the FcgammaRIIIa 158/V variant with RA in two diverse populations

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Rheumatoid Arthritis (RA) is typically associated with the presence of rheumatoid factors (RF), autoreactive immunoglobulins capable of complexing with IgG. Receptors for IgG may play a key role in clearance of immune complexes and the genes encoding Fcgamma-maR are potential candidates in RA.

A single nucleotide polymorphism (559 T/G) in FcgammaRIIIa results in an amino-acid substitution (Phe/Val) at position 158 which has functional significance: 158Val has a higher affinity for binding some IgG allotypes than 158Phe. Published studies of associations of this polymorphism with autoimmune diseases have given variable results.

To investigate this in RA, we have analysed this polymorphism in two genetically diverse populations: UK Caucasians (398 RA cases and 289 healthy controls) and Northern Indians (63 RA cases and 93 controls). Reliable typing of the 559 T/G polymorphism is greatly hindered by the high homology between FcgammaRIIIa and the neighbouring FcgammaIIIb, which has an invariant G at position 559. A rigorous review of the published typing methods revealed an average error rate of over 10% of genotypes obtained by a single method. The typing in this study was therefore done by complementing two different approaches: PCR-RFLP and allele specific PCR.

A significant reduction in the frequency of the rare GG genotype was seen among the Indian RA cases (RR=0.2 [0.05-0.7],  $P < 0.02$ ), although the allele frequencies were similar to those found in the control cohort (see table). Among the UK Caucasians, no significant differences were found for either the allele or genotype frequencies (the study had 95% statistical power to detect a genotype relative risk of 2 and an allelic association with OR of 1.6).

We conclude that the 158Phe/Val functional polymorphism in the FcgammaRIIIa gene does not predispose to RA in Caucasians, although there may be an effect among Indians. Further studies will be required to confirm this.

| UK Caucasian       | TT       | TG       | GG      | Phe | Val |
|--------------------|----------|----------|---------|-----|-----|
| Controls (n = 420) | 172(41%) | 213(51%) | 35(8%)  | 66% | 34% |
| RA cases (n = 401) | 165(41%) | 189(47%) | 47(12%) | 65% | 35% |
| Indian             | TT       | TG       | GG      | Phe | Val |
| Controls (n = 93)  | 44(47%)  | 35(38%)  | 14(15%) | 67% | 33% |
| RA cases (n = 63)  | 36(57%)  | 25(40%)  | 2(3%)   | 72% | 28% |

## P109

### The exchange of one single amino acid at position 71 of the DR4 β-chain leads to significant differences in antigen processing and presentation of a human autoantigen chaperoned by a member of the HSP70 family

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The immune response to protein antigens depends on processing by antigen presenting cells and subsequent presentation of peptides to specific T cells by molecules of the MHC. In rheumatoid arthritis (RA) there is much evidence implicating a positive genetic association of the disease with several alleles of the DRB1 gene, characterised by a common amino acid sequence at position 70-74 ("shared epitope"). The most frequent of these alleles is DRB1\*0401 with the sequence QKRAA, which is also present in the E. coli protein DnaJ. DnaJ and DnaK, a member of the HSP70 family, or their homologs in other species, together form an important chaperone machinery in bacteria and higher organisms, including man. It was proposed that the motif QKRAA might be involved in binding between DnaJ and DnaK, and also in binding of QKRAA-containing DR β-chains to the human heat shock cognate protein HSC70. We used a T cell clone with a restriction pattern similar to the genetic association of RA, being specific for an epitope of the α-chain of the human acetylcholine receptor (AChR). It reacted with synthetic peptides presented by murine P388.D1 expressing the human DRα and DRB1\*0401 (71Lys) or DRB1\*0408 (71Arg), with slight preference for 0408. In contrary, the recombinant α-chain (r1-437) or AChR obtained from human muscle extracts were much better presented by 0401. This preference depended entirely on the presence of E. coli DnaK or human HSC70 in the antigen preparations; the response was lost, if HSP70 molecules were removed, and reconstituted by their addition. We conclude that efficient processing of the long protein requires the presence of a member of the HSP70 family, which besides protecting the epitope, interacts intracellularly well with 0401, but less well with 0408 β-chains, and thus participates in the process of peptide loading. This mechanism might be of importance for immune responses against foreign antigens (advantage of DRB1\*0401) as well as against autoantigens (disadvantage of DRB1\*0401).

**P110**

**Time to lupus nephritis: impact of gender and ethnicity**

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**Objective:** There is a paucity of literature regarding the time to development of nephritis among SLE patients. Our goal was to define this important parameter for a large multi-ethnic cohort, with an emphasis on the impact of gender and ethnicity.

**Methods:** 779 SLE patients with disease satisfying the ACR criteria were classified as non-nephritis or definite nephritis patients based on questionnaire and comprehensive medical record review. Patients classified with definite nephritis fulfilled the criteria of proteinuria (> 0.5 g per 24 hrs), active sediment, or renal biopsy consistent with SLE. 716 (92%) were female. The ethnic distribution was: Caucasian 453 (58.2%), Hispanic 123 (15.8%), Asian 97 (12.4%), African American 75 (9.6%), and other 36 (4.6%). The annual rates of developing nephritis among different gender and ethnic subgroups were derived using Kaplan-Meier estimates.

**Results:** The table shows gender and ethnicity based Kaplan-Meier estimates of the proportion of patients with nephritis at designated time intervals. The curves are significantly different for males vs. females and Caucasians vs. non-Caucasians based on the log-rank test. The curves for Hispanic, Asian and African American subgroups did not differ significantly.

Proportion with nephritis at intervals after SLE diagnosis

|           | 1 year | 2 years | 3 years | 5 years | 10 years | P value *                |
|-----------|--------|---------|---------|---------|----------|--------------------------|
| Male      | .47    | .51     | .51     | .54     | .57      |                          |
| Female    | .20    | .23     | .24     | .25     | .30      | 8.16 x 10 <sup>-7</sup>  |
| Caucasian | .15    | .17     | .17     | .18     | .20      |                          |
| Non-Cauc  | .33    | .37     | .40     | .41     | .49      | 6.23 x 10 <sup>-14</sup> |

\* P value for log-rank test

**Conclusions:** These results are a significant contribution to the data regarding time to development of nephritis in SLE, and emphasize the important influence gender and ethnicity.

**P111**

**Increased apoptosis level in late stages of rheumatoid arthritis correlates with macrophage number**

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**Introduction:** Rheumatoid arthritis (RA is a chronic inflammatory disease characterized by synovial hyperplasia and excessive mononuclear infiltration. Altered apoptosis was proposed as a possible mechanism for cell accumulation. *In vitro* experiments showed that monokines are able to inhibit synovial apoptosis in a dose dependent manner. In this study we aim to investigate synovial apoptosis with respect to disease duration, inflammatory cell type and monokines expression.

**Materials and methods:** Synovial biopsy specimens from eleven patients with longstanding RA (mean disease duration 21 years)

and eight with early RA (mean disease duration 5 months) have been investigated. Samples were evaluated for apoptosis (TUNEL method combined with morphologic analysis), cell surface markers (CD3, CD68) and monokine expression (IL1 $\alpha$ , IL1 $\beta$ , TNF $\alpha$  and IL6). Tissue sections were then microscopically analysed using computerised image analysis. Statistical analysis was done using Mann-Whitney test, Spearman correlation test and linear regression.

**Results:** Apoptosis level in RA synovium is significantly higher in late cases compared with early ones ( $P = 0,001$ ), while macrophage population significantly decreases during disease progression ( $P = 0,003$ ). Macrophage score is negatively correlated with apoptosis level ( $R = -0,618$ ;  $P = 0,0088$ ). In contrast, no correlation could be observed between apoptosis and monokine expression or T cell score.

**Discussion:** Low level of apoptosis in early RA cases suggests an ineffective cell death mechanism that ultimately contributes to cell accumulation into the joint and propagation of the inflammatory response. Apoptosis is restored during disease progression, in parallel with a decrease of the macrophage number. These findings suggest apoptosis as a possible marker for early RA and a promising therapy target.

**P112**

**Protein kinase C inhibition dephosphorylates the ribosomal P proteins while inducing apoptosis in Jurkat cells and peripheral human T cells**

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The control of phosphorylation of CK2 target sites has long been a matter of controversy. We have demonstrated that phosphorylation of the ribosomal P protein CK2 sites is reliably measurable by 2D western blotting of whole cell lysates. Physiologically, phosphorylation of the CK2 sites of the P proteins is necessary for the elongation phase of protein translation, which can be used as an indirect parameter of P protein function. Based on previous data showing that crosslinking of CD95 and hyperthermia lead to the dephosphorylation of the ribosomal P protein CK2 phosphorylation sites and decreased protein synthesis, which was associated with the induction of apoptosis in Jurkat cells, we examined whether similar mechanisms are initiated when apoptosis is induced by inhibition of the PKC pathway. In Jurkat cells and freshly isolated peripheral blood T cells, rapid dephosphorylation of the ribosomal P proteins P0, P1 and P2 was induced by low concentrations of chelerythrine, a specific inhibitor of PKC. Neither of the specific PKC activators thymeleatoxin or PMA were able to prevent the dephosphorylation. Inhibition of intracellular Ca<sup>2+</sup> release by TMB-8 also induced dephosphorylation of the P proteins, which is compatible with the requirement of intracellular Ca<sup>2+</sup> for classical PKC isozyme activity. Chelerythrine also induced apoptosis in Jurkat cells, which was prevented by zVAD-fmk and ZnCl<sub>2</sub>, though these agents did not inhibit the dephosphorylation of the P proteins. The effects of chelerythrine were not due to altered CK2 activity, and there was no evidence that the cAMP-dependent PKA, ornithine decarboxylase, or protein phosphatase 2A pathways were involved in signaling leading to P protein dephosphorylation. The dephosphorylation of the P proteins was accompanied by markedly reduced whole cell protein synthesis, which, in parallel with dephosphorylation of the P proteins, was not affected by zVAD-fmk or ZnCl<sub>2</sub>. Freshly isolated peripheral blood T cells showed the same pattern of responses as Jurkat cells, with the exception that chelerythrine did not induce apoptosis in resting T cells.

Our results demonstrate that inhibition of Ca<sup>2+</sup> dependent PKC activity decreases the phosphorylation of the P protein CK2 sites and protein synthesis. The failure of caspase inhibition to prevent the dephosphorylation and decreased protein synthesis due to PKC inhibition indicates early divergence of PKC and caspase-dependent signaling in T cells and Jurkat cells.

## P113

### Nucleosome binding by serum amyloid P component from SLE patients

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There is evidence in favor of apoptosis dysfunction being involved in the pathogenesis of SLE. Since mice deficient in the nucleosome-binding protein: serum amyloid P component (SAP) develop antinuclear immunity and nephritis it is possible that impaired clearance of nuclear material from apoptotic cells is the key defect. We therefore developed a solid-phase assay for the binding of SAP to nucleosomes and investigated the nucleosome binding characteristics of SAP from 20 different SLE patients as compared with normal donors. We could not demonstrate any significant differences between the groups and therefore the functions of other pentraxins (such as C-reactive protein) or DNA binding molecules (such as C1q) will be examined in future studies.

## P114

### Nucleosome is a necrosis inducer for lymphocytes: consequences in systemic lupus erythematosus

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Nucleosome is a major autoantigen in systemic lupus erythematosus. It is composed of DNA (multiple of 180 bp) and the five histones H1, H2A, H2B, H3 and H4. Previous works have shown the presence of circulating nucleosome in sera of lupus patients, which could be due to increased apoptosis or impaired phagocytosis, both resulting in secondary necrosis and release of nucleosome. On the other hand, it was shown that nucleosome could bind to the surface of cells, such as lymphocytes. In order to better understand the role of this circulating complex, we analysed the effect of purified nucleosome on living murine lymphocytes. Here we show that nucleosome induces necrosis of cells, and not apoptosis, as assessed by different techniques. Similar results were obtained with mono-, di-, tri- and poly-nucleosomes. Moreover, this effect is time and dose dependent, is impaired when nucleosome is heat-inactivated and is not observed with other non related purified proteins. Finally, we analysed in more details the sensitivity of B and T cells to nucleosome. These results suggest that nucleosomes released by apoptotic cells could induce necrosis of neighbouring cells, thus allowing the release of cell contents in high amounts. This phenomenon would act as an "amplification loop" and could explain how the peripheral tolerance is broken and is in agreement with inflammatory responses which are normally not associated with apoptosis.

## P115

### Anti-Fcγ receptor (FcγR) autoantibodies (Ab) delay apoptosis of polymorphonuclear cells (PMN) in systemic autoimmune diseases by inducing the production of G-CSF and GM-CSF

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FcγRIIIb is expressed by PMN, and cell-free receptor has also been found circulating in body fluids. We have examined 222 patients with rheumatoid arthritis (RA), systemic lupus erythematosus and primary Sjögren's syndrome and classified anti-FcγR auto-Ab into three groups, based on the results of an indirect immunofluorescence (IIF) test and an ELISA : IIF+/ELISA+ (group A), IIF+/ELISA- (group B) and IIF-/ELISA+ (group C). PMN-binding Ab, *i.e.* groups A and B, prolonged the survival of PMN by delaying spontaneous apoptosis, and reduced adhesiveness and respiratory burst of these cells. Interestingly, recombinant non-glycosylated and purified glycosylated FcγR produced similar effects as the related auto-Ab.

To delineate the mechanism(s) by which the PMN life span is prolonged, we studied the response of PMN to F(ab')<sub>2</sub> fragments of anti-FcγR (CD16) monoclonal Ab. CD16 engagement decreased apoptosis and prevented the up-regulation of β2 integrins, particularly CD11b which is the α chain of complement receptor 3, and CD18 which is its β chain.

The expression of mRNA for G-CSF and GM-CSF was induced. Release of these chemoamines followed CD16 stimulation, suggesting an autocrine involvement of survival factors in the rescue of PMN. The levels of G-CSF and GM-CSF paralleled the amounts of CD16 monoclonal Ab used to stimulate the cells. This set off a partial reduction of caspase 3 activity and was associated with down-expression of *Bax*. It was shown that anti-G-CSF and anti-GM-CSF Ab inhibited these events, while anti-TNFα was ineffective.

Overall, apoptosis of aged PMN can be modulated by signalling through FcγR, which may occur in patients with PMN-binding auto-Ab. This might be particularly relevant in the synovial fluid of patients with RA.

## P116

### Phage display as a tool to study human autoantibodies and autoantigens in systemic autoimmune disease. Selection of recombinant (auto)-antibodies specific for human autoantigens in rheumatic disease (RA, SLE, SSc) from human autoimmune-patient and immunized chicken derived phage display libraries

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One important property of the immune system is its ability to discriminate between self and non-self antigens. The unresponsiveness of the immune system to self-antigens is called self-tolerance, loss of this property results in immune reactions against own or autologous antigens. Such reactions often are associated with an autoimmune disorder and eventually may contribute to clinical manifestations. The humoral immune response plays a crucial role in the

onset of autoimmunity, and it is a general observation that autoimmune diseases are associated with distinct profiles of autoantibodies. During the last five years there has been a growing interest in finding possible connections between apoptosis and autoimmunity. It has been hypothesized that the recognition, uptake, processing, or presentation of modified self-antigens may promote autoantibody production. At present, many autoantigens have been found that are modified (i.e. cleaved, phosphorylated or dephosphorylated) during apoptosis. Using autoimmune patient sera in immunoprecipitation and Western blotting assays with cell extracts derived from non-apoptotic and apoptotic cells, we identified sera reactivities specific for (novel) modifications of autoantigens.

In this paper we give an overview of our studies on human recombinant autoantibodies derived from patients suffering from rheumatic diseases. Furthermore, we describe the generation of recombinant chicken antibodies specific for human autoantigens that will also be used to study these antigens and (apoptotic) modifications thereof in more detail.

We selected human recombinant antibodies from patient phage display scFv combinatorial antibody libraries (complexity of 107 or higher) derived from peripheral blood or bone marrow lymphocytes of patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and scleroderma (SSc). Next to the human patient libraries we also used chicken libraries made from spleens of animals immunized with 7 human (auto)antigens simultaneously. Screening of both patient and animal derived libraries with recombinant or purified autoantigens resulted in several recombinant monoclonal human and chicken (auto)-antibodies. From our SLE patient libraries, autoantibodies against U1snRNP components U1-A, U1-C, U1-70k and U1-RNA were selected. Moreover, from our SLE libraries we selected anti Ro52 and anti ribosomal P protein antibodies, and from both our SLE and scleroderma libraries we selected anti-La and anti-a-fodrine recombinant antibodies. From our RA patient libraries antibodies specific for RA related peptides were obtained. All human scFv clones obtained were characterized by ELISA, immunoprecipitation assays, western blotting, epitope mapping and of some clones the affinities were measured and competition experiments with patient sera were performed. Sequence analysis was performed to study the germline usage. All chicken clones were sequenced and analyzed by LIA, Western blot and ELISA.

We will discuss characteristics of some of the selected scFv's i.e. epitope mapping, germline gene usage, and competition experiments with patient sera.

The phage autoantibodies selected from autoimmune patient libraries were also analyzed for their specificity for (apoptotically) modified forms of their target autoantigens by Western blotting and immunoprecipitation assays using apoptotic cell-extracts. Some anti-La, anti-70K and anti-RA peptide scFv's recognized (apoptotically)-modified forms of their target antigens.

**Conclusions:** The use of antibody phage display proves to be an extremely helpful technique in studying autoantibodies and autoantigens. Modifications of autoantigens (by apoptosis and/or necrosis) seem to play a major role in the ontogeny of autoimmune diseases. Currently, by using antibody phage display libraries in combination with patient sera we continue our search for possible modifications of autoantigens involved in the ontogeny of autoimmune disease.

## P117

### Diagnostic value of synovial fluid analysis in pigmented villonodular synovitis (PVS)- a proposal of diagnostic criteria.

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PVS is an idiopathic lesion that affects the synovial joints, tendons, sheaths and bursa through the production of tumour-like growths. Diagnosis of PVS is difficult. Arthroscopy and biopsy together with microscopic examination are usually a base. According to our experience, cytologic features of synovial fluid are a very useful diagnostic tool in PVS.

**Material and methods:** Synovial fluids (SF) from the joints of 14 patients with biopsy-proven PVS were examined. Moreover in all the patients features of the disease were confirmed in surgical specimens. Synovial fluids were divided into three samples, for physico-chemical analysis, bacteriological and cytologic findings, and placed in sterile tubes. For cytological examination MGG staining was used.

**Results:** SF analysis had revealed an inflammatory character of effusion. In all the cases synovial fluids were bloody and fragments of synovial villi in their sediments were observed as well as multinucleated giant cells, pseudomalignant cell, macrophages with phagocytized hemosiderin, foam cell and a few synoviocytes. Moreover the fat crystals were seen, under polarized light.

**Conclusion:** Cytological features of synovial fluid in PVS are in parallel with results of microscopic examination of joints tissues. In our opinion SF analysis should be the first step in the diagnostic procedure in PVS. We propose the following criteria:

*Major:*

1. the presence of bloody fluid with fragments of synovial villi in sediment
2. macrophages containing hemosiderin
3. pseudomalignant cells

*Minor:*

1. multinucleated giant cells
2. foam cells
3. fat crystals

The diagnosis of PVS can be established if all the major and at least one of the minor criteria are fulfilled.

## P118

### Analysis of anti-Ro52 antibodies in sera of healthy subjects

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Anti-Ro/SSA antibodies (ab) are directed to two proteins, Ro60 and Ro52. While anti-Ro60 ab are predominantly found in patients with SLE or primary Sjögren's syndrome, anti-Ro52 ab can be detected also in sera of healthy subjects. These antibody escape detection by ELISA or immunoblotting and can be found only in immunoprecipitation assays. Recently, an unexpected interaction of Ro52 with IgG has been reported which appeared to occur independently of the antigen binding site. To investigate this unusual interaction, serum IgG was covalently bound to protein A sepharose (PAS) or an anti-IgG agarose column was used and incubated with HeLa cytoplasmic extracts and various Ig fractions as competitors (IgG1-4, IgM, Fab, F(ab)2, Fc, pFc', Fc', C1q). Proteins bound to



immobilized IgG were detected by immunoblotting using anti-Ro/La positive patients sera and a monospecific anti-Ro52 antibody for identification of bound proteins.

In these assays Ro52 only was bound to the column-IgG complex. Binding of Ro52 was inhibited by total IgG and Fc, but not Fab, F(ab)2 or C1q.

In another experiment IgG anti-IgM was complexed with PAS and Ro52 binding was demonstrated. This binding was partly inhibited by IgM, suggesting an unspecific interaction of Ro52 with the IgG-Fc.

This antigen/antibody reaction is seen in all IgG subclasses binding to the PAS, namely IgG1, IgG2 and IgG4, with IgG2 reacting very strongly with the Ro52.

In conclusion, these data show an interaction of Ro52 with all types of IgG of healthy persons. This interaction appears therefore to be substantially different from normal antigen-antibody interaction. The biological function of this interaction is unclear.

## Abstracts of invited lectures

### L1

#### The impact of DNA chip technology on molecular medicine

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The recent popularity of DNA chip technology has been fostered by the increasing demand for new tools, which allow the simultaneous analysis of large numbers of nucleic acid hybridization experiments in a timely fashion. The development of DNA chip-based assays has been strongly driven by modern approaches aiming at the comprehensive analysis of multiple gene mutations and expressed sequences. The broad range of current DNA chip applications include the detection of pathogens, the measurement of differences in the expression of genes between different cell populations as well as the analysis of genomic alterations such as sequence or copy number alterations of disease related genes or single nucleotide polymorphisms. A brief overview of the impact of DNA chip technology on the field of Molecular Medicine will be provided, followed by a more detailed presentation on DNA chip technology for large-scale differential expression profiling.

### L2

#### Protein microarray characterization of the autoantibody response in systemic lupus erythematosus and related diseases

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Systemic lupus erythematosus (SLE) is the prototypical systemic autoimmune disease characterized by production of autoantibodies against a wide range of nuclear self-antigens. These antigens include DNA, histone and several constituents of the RNA splicing complex. Binding of autoantibodies to their specific antigens leads to tissue injury, ultimately resulting in end-organ damage. Individual SLE patients are known to have considerable variability in their specific autoantibody response patterns, and this in part correlates with their clinical manifestations. We have refined protein microarray technology and utilized it to study variation in the autoantibody response between individual SLE patients. We have further

extended these studies to other diseases including primary biliary cirrhosis, rheumatoid arthritis, scleroderma, mixed connective tissue disease, multiple sclerosis, diabetes mellitus, and the myositides. Protein microarrays are produced by the application of thousands of proteins and peptides to the surface of a glass microscope slide using a robotic arrayer. We have developed an array containing the major SLE antigens including dsDNA, ssDNA, Sm, Ro, La, histones, and Sm/RNP, along with several other common disease-specific autoantigens. Arrays are probed with serum from disease or control patients, followed by incubation with fluorescently labeled, anti-human secondary antibody. Our array analysis reveals distinct autoantigen response patterns in individual SLE patients. ELISA, immunoprecipitation, and western blot analysis validate our array results. Protein autoantigen arrays represent a powerful tool which may be used to perform comprehensive studies of the breadth of epitope spreading, as well as the fine specificity of the autoantibody response. The large scale of our protein arrays allows us to examine reactivity patterns against a much wider range of autoantigens than was previously possible with more traditional methods. Our ability to distinguish autoantibody specificity patterns using autoantigen microarrays will provide further insight into the role of autoantibodies in disease progression and pathogenesis in subsets of SLE patients with distinct autoantibody responses.

### L3

No abstract

### L4

No abstract

### L5

#### The integration of functional genomics, combinatorial chemistry and nanotechnology into a miniaturized drug discovery process

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Future concepts in miniaturized HTS screening technologies will concentrate on making optimal use of the two emerging technologies, combinatorial chemistry and functional genomics. To effectively exploit compounds from highly-parallel combinatorial synthesis and the high number of new target proteins from functional genomics, Novartis and Evotec BioSystems developed the CONA-BSP (confocal nanoscanning – bead scanning picking technology) as a novel high throughput – low hit-rate HTS process. In combination with the Novartis proprietary AIDA-Technology, quantitative on-bead confocal fluorescence screening can be combined with off-bead confirmation via a series of fluorescence techniques such as fluorescence anisotropy, or rotational correlation time applied to equilibrium binding studies.

Single molecule fluorescence spectroscopy and confocal nanoscanning/AIDA technology provide an optimal combination for a miniaturized automated uHTS process with high mechanistic resolution in functional and coupled assay systems.

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The integration of single molecule detection technologies into miniaturized drug screening: Current status and future perspectives.

C. Bühler, K. Stöckli, and M. Auer

Review in press: *Fluorescence Spectroscopy: Valeur/Brochon, Trends in Fluorescence Spectroscopy* (Springer Verlag, 2001).

## L6

### DNA therapeutics: a feasible option for treatment of inflammatory diseases?

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Recombinant proteins can be very potent, but their therapeutic application can be strongly hampered by inappropriate distribution, dosage, kinetics or toxic effects. Targeted delivery of proteins such as cytokines would be strongly desirable.

DNA therapeutics comprise the delivery of genetic information on a piece of DNA as therapeutic prodrug. This prodrug can be transcribed and translated into a protein (the actual drug) within the target cells, preferably in a tissue-specific and bio-regulated fashion. Basically, two types of nonviral gene transfer systems [1] have been developed: particle-based systems, with DNA packaged into cationic lipids or polymers; and physical techniques which are based on combining DNA with a physical device. Intramuscular administration of naked DNA has already proven as interesting concept for vaccination [2], despite the low efficiency of the method. Two physical device technologies, electroporation and the gene gun, were found to enhance gene expression levels up to 1000-fold over injection of naked DNA alone. This enhancement has also recently been shown by several groups to trigger immune responses against defined antigens in several species [3].

We have generated particle-based systems that can target gene delivery and expression into distant target tissues. We use DNA polyplexes conjugated with cell-binding ligands such as transferrin for receptor-mediated endocytosis. The surface charge of complexes is masked by covalent coating with polyethylenglycol (PEG). Tumor targeting has been demonstrated in mouse models after systemic administration. With systemically applied tumor necrosis factor (TNF) alpha gene, tumor necrosis and regression of tumors was observed, but no systemic TNF-related side effects. Opportunities to apply local or systemic DNA therapeutics for inflammatory diseases will be discussed.

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## L7

### Chromosome segregation one hundred years after Mendel's rediscovery

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In eukaryotic cells, replicated DNA strands remain physically connected until their segregation to opposite poles of the cell during anaphase. This "sister chromatid cohesion" is essential for the alignment of chromosomes on the mitotic spindle during metaphase. Cohesion depends on a multisubunit protein complex called cohesin, which possibly forms the physical bridges that connect sisters. Proteolytic cleavage of cohesin's Scc1 subunit at the metaphase to anaphase transition is essential for sister chromatid separation and depends on a conserved protein called separin. We show here that separin is a cysteine protease related to caspases and that it alone can cleave Scc1 in vitro. By replacing one of Scc1's cleavage sites by that for a different site specific protease, we show that cleavage of Scc1 in metaphase arrested cells is sufficient to trigger the separation of sister chromatids and their segregation to opposite cell poles.

## L8

### Genetic analysis of the pentraxin genes in SLE

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The aetiology of systemic lupus erythematosus (SLE) is unknown. However, there is good evidence to support a genetic contribution in lupus, including a number of mouse strains that are genetically predisposed to develop lupus. Several groups have published genome-wide mapping studies on multi-case families. More than 15 intervals have been linked with SLE – they are large enough to contain several hundred genes; the aetiological polymorphisms contained within them remain to be established.

We are establishing a large collection of single case nuclear families with the aim of fine mapping the aetiological polymorphisms. Using a candidate gene approach, we have examined several genes, which lie within the linked intervals. First, we identified genetic markers in the candidate genes. The inheritance of the markers in our nuclear families was then tested using the program TRANSMIT which compares the observed and expected rates of transmission of marker alleles (or haplotypes) from parents to offspring. A marked distortion away from random segregation indicates association with disease.

We have hypothesised that genetic variation in the pentraxin genes, C-reactive protein (CRP) and serum amyloid component P (SAP) predisposes to SLE. These two genes are tightly linked on chromosome 1q21-23, a region linked to human SLE. Other evidence implicating these includes the defective CRP response in SLE and the presence of antinuclear autoimmunity in *Sap* knockout mice. We identified five novel single base pair polymorphisms (three in *CRP* and two in *SAP*) and tested these for evidence of association. Individuals from 354 families were studied.

**Table. Transmission of Markers across CRP and SAP to SLE Probands**

| Marker     | Allele | Observed | Expected | Chi square | P value |
|------------|--------|----------|----------|------------|---------|
| CRP C1122T | 1      | 461      | 460      | 0.01       | > 0.05  |
|            | 2      | 183      | 183      |            |         |
| CRP G1979A | 1      | 416      | 428      | 2.6        | > 0.05  |
|            | 2      | 222      | 219      |            |         |
| CRP G808C  | 1      | 359      | 361      | 0.74       | > 0.05  |
|            | 2      | 25       | 22       |            |         |
| SAP G-246A | 1      | 490      | 491      | 0.06       | > 0.05  |
|            | 2      | 146      | 144      |            |         |
| SAP G902T  | 1      | 354      | 356      | 0.47       | > 0.05  |
|            | 2      | 28       | 26       |            |         |

These data provide no evidence for a genetic contribution to human SLE from the pentraxin genes. When haplotypes across this locus were examined there was similarly no evidence of association. The defective CRP response in human SLE is unlikely to be related to variation at the *CRP* locus itself.

## L9

### The role of DNA in the pathogenesis of SLE

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Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease characterized by antibodies to DNA. These antibodies serve as markers of diagnosis and prognosis as well as serological markers of disease pathogenesis. While designated as autoantibodies, SLE anti-DNA target sites that are widely conserved among DNA of both self and foreign origin. This crossreactivity, a feature that appears common among SLE antinuclear antibodies, raises the possibility that immune responses to DNA may arise from stimulation by foreign DNA. This possibility has gained credence from observations that DNA from bacteria has potent immunological properties. These properties include polyclonal B cell activation and induction of cytokines such as IL-12. Furthermore, sera of normal human subjects contain anti-DNA antibodies which selectively bind to DNA from certain bacterial species. Immunization experiments in mice fully support the possibility that bacterial DNA can initiate or sustain SLE anti-DNA production. Recently, studies in mice have demonstrated that self DNA has immunological activity and is not inert as has been widely assumed. As shown in *in vitro* experiments, DNA from mammalian species including human and bovine, can inhibit cytokine production induced by bacterial DNA and, in certain systems, the cytokine response to LPS. As such, self DNA may play a regulatory role in immunity, inhibiting response in settings of tissue inflammation or destruction where self antigens are released from cells. These considerations suggest that anti-DNA responses in SLE could result from a crossreactive response to foreign DNA or an aberrant response to self DNA in which the inhibitory signals of mammalian DNA are insufficient or overcome. In either instances, models of SLE must take into account that DNA plays an active role in immune responses and, depending upon species and base composition, may be stimulatory or inhibitory.

## L10

### Gene expression analysis of Th1 and Th2 cells: clues to homing in inflammation

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Many pathological processes, including rheumatoid arthritis, are associated with the presence of specialised subsets of T helper

cells at the site of inflammation. Understanding the genetic program that control the functional properties of Th1 versus Th2 cells may provide insight into the pathophysiology of inflammatory diseases. We compared the gene expression profiles of human Th1 and Th2 cells using high-density oligonucleotide arrays with the capacity to display transcript levels of 6000 human genes. This approach resulted in the identification of more than 200 differentially expressed genes, including genes controlling the different steps of lymphocyte migration and homing<sup>1</sup>. A subset of these genes was further upregulated by exposure of differentiated Th1 cells to IL-12. Functional assays and *in vivo* expression of selected genes have validated the biological relevance of this study. Our results provide novel insight into the transcriptional program controlling the differential ability of T helper subsets to traffic and localise to sites of inflammation.

<sup>1</sup>Rogge et al. 2000. Nature genetics 25:96-101

## L11

No abstract

## L12

### From perinuclear factor to citrulline, a target structure for autoantibodies in rheumatoid arthritis

G Serre

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Antiperinuclear factor (APF) and the so-called "antikeratin antibodies" (AKA), have been described 37 and 22 years ago, respectively. Now, their diagnostic value in Rheumatoid Arthritis (RA) was confirmed on thousands of patients.

In the past few years the molecular targets of these RA-specific serum IgG antibodies were identified : first those of AKA were shown to be acidic variants of filaggrin in human epidermis and (pro)filaggrin-related proteins in rat oesophagus epithelium ; secondarily the target of APF in human buccal mucosa cells was demonstrated to correspond to tissue-specific forms of (pro)filaggrin. APF and AKA were proved to constitute two overlapping subgroups of antibodies belonging to a same family of antifilaggrin autoantibodies (AFA).

All these AFA-targetted proteins were demonstrated to be deiminated i.e. having their arginyl residues (arginines involved in peptidic bonds) transformed into citrullyl residues (citrullines). This post-translational enzymatic modification is due to a peptidyl-arginine deiminase. AFA are unreactive with native recombinant human filaggrin but become highly reactive after enzymatic deimination of the protein. Moreover synthetic peptides derived from the human filaggrin sequence are reactive with AFA only when their arginyl residues have been substituted by citrullyl residues (citrullinated peptides). Therefore AFA are directed to peptidic epitopes in which citrullyl residues play a pivotal role, nevertheless the neighbouring residues are important since certain are permissive and participate to generation of AFA epitopes whereas others are non-permissive and make the reactivity with AFA impossible.

Identification of the molecular targets of AFA allowed the development of new tests for their detection, using either (pro)filaggrin extracted from epithelia or deiminated recombinant filaggrins and/or filaggrin-derived citrullinated peptides. Several of these new tests prove to be highly efficient in the diagnosis of RA and largely more performant than the reference APF and AKA tests. They show that at least 2 out of 3 RA patients develop the very specific antifilaggrin B autoimmunity.

In rheumatoid synovial tissue, (pro)filaggrin was confirmed to be absent, however several deiminated proteins were detected. Among them, only two proteins were highly reactive with AFA. They were identified as the alpha and beta chains of fibrin. Deiminated fibrin therefore appears as the major synovial target of AFA and probably correspond to their genuine target.

In RA patients, the proportion of AFA among IgG was recently found to be largely higher in the synovial interstitium than in synovial fluid and serum, moreover AFA were shown to be secreted by plasma cells of the rheumatoid pannus.

These results strongly suggest that the chronic conflict between the locally secreted AFA / antifibrin autoantibodies and the fibrin deposits particularly prominent in the RA synovium, play a central role in the pathophysiology of RA.

### L13

#### Anti-inflammatory activity of statins: potential use in the anti-phospholipid syndrome

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**Background:** Hydroxymethylglutaryl Coenzyme A reductase (HMGCoA-red) inhibitors are cholesterol lowering drugs which display pleiotropic effects on several cell types including endothelial cells (EC). Patients with antiphospholipid syndrome (APS) are characterized by the persistent presence of antiphospholipid antibodies (aPL) and by a high incidence of recurrent thrombotic events. aPL have been demonstrated to bind and activate cultured human EC thus contributing to a prothrombotic state. We evaluated the ability of HMGCoA inhibitors to affect the EC activation induced *in vitro* by aPL and in particular by antibodies reacting with the PL-binding protein  $\beta$ 2 glycoprotein I ( $\beta$ 2GPI). Both human monoclonal IgM and polyclonal IgG anti- $\beta$ 2GPI antibodies were used. EC activation was evaluated as adhesion molecule (ADM) expression and cytokine production.

**Methods:** ADM expression was evaluated by a cell ELISA. EC were incubated with human recombinant (hr) IL-1 $\beta$  (50 U/ml), hr TNF $\alpha$  (10 ng/ml), LPS (20 ng/ml) or with human anti- $\beta$ 2GPI antibodies (100  $\mu$ g/ml) for 4 hr for E-Selectin expression and for 20 hr for ICAM-1 evaluation. Cytokine production was investigated by using the RiboQuant<sup>TM</sup> *in vitro* transcription assay to measure IL-6 mRNA expression. As control, EC monolayers were incubated with irrelevant monoclonal or polyclonal antibodies or medium alone. The same experiments were carried out with EC monolayers pre-incubated overnight with fluvastatin or simvastatin (1-10  $\mu$ M) in the absence or presence of mevalonate (100  $\mu$ M). E-Selectin specific NF $\kappa$ B expression was also evaluated by the gel-shift assay.

**Results:** Both statins inhibited in a concentration dependent-manner the ADM expression induced by anti- $\beta$ 2GPI antibodies as well as those induced by the other agonists, being fluvastatin more efficient than simvastatin. Fluvastatin also down-regulated the mRNA expression specific for IL-6 and significantly inhibited E-Selectin NF $\kappa$ B DNA-binding. The simultaneous addition of mevalonate to fluvastatin completely prevented the drug inhibitory effect.

**Conclusions:** These data demonstrates for the first time that statins (and particularly fluvastatin) are able to inhibit an endothelial pro-adhesive and pro-inflammatory phenotype induced by different stimuli including anti- $\beta$ 2GPI antibodies or pro-inflammatory cytokines. Altogether these findings suggest a potential usefulness for statins in the prevention of the APS pro-atherothrombotic state.

### L14

No abstract

### L15

No abstract

### L16

#### The place of mitochondria in apoptosis

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Apoptosis research has recently experienced a change from a paradigm in which the nucleus determined the apoptotic process to a paradigm in which caspases and, more recently, mitochondria constitute the center of death control. Mitochondria undergo major changes in membrane integrity before classical signs of cell death become manifest. These changes concern both the inner and the outer mitochondrial membranes, leading to the dissipation of the inner transmembrane potential and/or the release of intermembrane proteins through the outer membrane. An ever increasing number of endogenous, viral, or xenogeneic effectors directly act on mitochondria to trigger permeabilization. At least in some cases, this is achieved by a direct action on the permeability transition pore complex (PTPC), a multi-protein ensemble containing proteins from both mitochondrial membranes which interact with pro- and anti-apoptotic members of the Bcl-2 family. At present, it is elusive whether opening of the PTPC is the only physiological mechanism leading to mitochondrial membrane permeabilization. Proteins released from mitochondria during apoptosis include caspases (mainly caspases 2, 3 and 9), caspase activators (cytochrome c, hsp 10, Smac/DIABLO), as well as a caspase-independent death effector, AIF (apoptosis inducing factor). Apoptosis inducing factor (AIF) is encoded for by one single gene located on the X chromosome. AIF is ubiquitously expressed, both in normal tissues and in a variety of cancer cell lines.

The AIF precursor is synthesized in the cytosol and is imported into mitochondria. The mature AIF protein, a flavoprotein (prosthetic group: FAD) with significant homology to plant ascorbate reductases and bacterial NADH oxidases, is normally confined to the mitochondrial intermembrane space. In a variety of different apoptosis-inducing conditions, AIF translocates through the outer mitochondrial membrane to the cytosol and to the nucleus. Ectopic (extra-mitochondrial) AIF increases the permeability of the outer mitochondrial membrane, thereby triggering the release of the caspase activator cytochrome c. Moreover, AIF induces nuclear chromatin condensation, as well as large scale (~50 kbp) DNA fragmentation. Thus, similar to cytochrome c, AIF is a phylogenetically old, bifunctional protein with an electron acceptor/donor (oxidoreductase) function and a second apoptogenic function. In contrast to cytochrome c, however, AIF acts in a caspase-independent fashion. The molecular mechanisms via which AIF induces apoptosis, as well as the phenotype of AIF knock-out cells will be discussed.

### L17

#### New approaches to inhibiting TNF production in rheumatoid arthritis: is pathological TNF regulated in the same way as protective TNF?

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The success of antiTNF therapy of rheumatoid arthritis with infliximab (Remicade) and etanercept (enbrel) has prompted us to seek other ways of inhibiting TNF production, and to seek to determine the cellular and molecular mechanisms underlying the excess and prolonged TNF synthesis in RA.

We have studied spontaneous synovial TNF production and found it to depend on the function of synovial T cells. These T cells behave like cytokine activated T cells and not antigen activated T cells from normal individuals. This was determined by comparing the TNF response to inhibitors of PI3Kinase and of NFkB in Dayer type Tcell-macrophage cocultures, using the 3 types of T cells.

This result has important implications, at several levels. First, it ends the controversy concerning the role of T cells in late RA, they are involved, but their function is atypical. Second, it demonstrates that the synovial T cells which resemble cytokina activated T cells are a goog target for therapy. As these cells are not present in acute protective immune responses, it predicts that if it turns out that the risk of infection increases with prolonged use of TNF inhibitors, targeting TNF indirectly by this approach, for example with a monoclonal antibody, might be a safer approach.

## L18

### Cytokine signalling: new insights and new opportunities for therapeutic intervention?

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It is well documented that cytokines have critical functions in regulating immune responses and remarkably, the number of cytokines continues to expand. One large family factors that includes many interleukins and interferons binds related receptors termed the Type I and Type II families of cytokine receptors. These receptors activate Janus kinases (Jaks) and Stat family of transcription factors. The essential and specific function of Jaks and Stats is particularly well illustrated by human and mouse mutations. For instance, mutations of human Jak3 results in severe combined immunodeficiency. These mutations are of interest in that they provide clues to Jak structure/function. Additionally, patients with mutations that allow for partial expression of the protein may have nonclassical clinical presentations in which autoimmune features are prominent. There are also a number of mechanisms by which cytokine signaling is attenuated. One important family of inhibitory molecules is the SOCS family. The possibility that the various components of the cytokine signaling pathway could be targeted to produce novel immunosuppressive compounds will be discussed.

## L19

### The pathogenesis of vasculitis

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In the secondary vasculitides, associated with infectious disorders, connective tissue diseases and other conditions, immune complexes play a major immunopathogenic role. Immune complexes are absent in most of the primary vasculitides. T-cells are, probably, involved in the large vessel vasculitides, particularly giant cell arteritis, whereas the small vessel vasculitides are associated with anti-neutrophil cytoplasmic autoantibodies (ANCA). Clinical observations, in vitro experimental findings, and in vivo data from animal experiments suggest that ANCA in those diseases, which are directed to proteinase 3 (PR3) or myeloperoxidase (MPO), are involved in their pathogenesis.

Most *in vitro* studies have focussed on ANCA-induced neutrophil activation. More recently the interaction between ANCA, neutrophils and endothelial cells has been studied in flow systems. ANCA appear to activate integrin-mediated adhesion of neutrophils and

adhesion-dependent degranulation. ANCA-induced monocyte activation has been studied to a lesser extent. The role of ANCA-specific T-cells is still under investigation. Epitope analysis showed T-cell reactivity to peptides from PR3 but no specific PR3 sequence could be identified that was preferentially recognized by T-cells of vasculitis patients compared to controls. *In vivo* experimental studies, in which an MPO-directed autoimmune response is generated, show the phlogistic potential of this response. Apoptotic neutrophils may, under certain circumstances, induce the induction of ANCA. Data from clinical and experimental studies suggest that ANCA alone are not sufficient to induce disease. Exogenous factors, in particular carriage of *Staphylococcus aureus* and silica exposure, may be involved as well. *S. aureus* products may elicit antibody responses resulting in focal immune complex deposition, e.g. in the kidneys. ANCA may aggregate the inflammatory response resulting in destruction of complexes and the development of severe necrotizing glomerulonephritis without immune deposits.

Taken together, the interplay between genetic and exogenous factors may induce autoimmunity to myeloid enzymes which, in concert, lead to the clinical expression of the ANCA-associated vasculitides.

## L20

### The pathogenesis of osteoarthritis: potential targets for therapy

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It is likely that the excessive production of cytokines, inflammatory mediators and growth factors by the inflamed synovium and activated chondrocytes play an important role in the pathophysiology of osteoarthritis. IL-1b and TNF-a can stimulate their own production and induce chondrocytes and synovial cells to produce other cytokines such as IL-8, IL-6, LIF, as well as stimulate proteases, nitric oxide (NO) and prostaglandin E2 (PGE2) production. NO and PGE2 are spontaneously produced by human osteoarthritis-affected cartilage. The excessive production of nitric oxide inhibits matrix synthesis and promotes its degradation. Furthermore, by reacting with oxidants such as superoxide anion, nitric oxide promotes cellular injury and renders the chondrocyte susceptible to cytokine-induced apoptosis. Although PGE2 is the predominant eicosanoid produced by OA cartilage, PGI2, PGD2, TXA2 and LTB4 are also spontaneously produced. These specific eicosanoids exert diverse effects on matrix metabolism and gene expression that require detailed elucidation. Differential gene product analysis also reveals increased expression of osteopontin (OPN) and fibronectin (FN) mRNA in human osteoarthritis-affected. Osteopontin inhibits the spontaneous production of inflammatory mediators such as NO and PGE2. Therefore, inflammatory and anti-inflammatory molecules produced by OA chondrocytes can be targeted in future therapeutic strategies of OA.

## L21

### The role of "nurse-like cells" in bone resorption observed in patients with RA

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Synovial stromal fibroblastic cells were histologically suggested to be derived from the mesenchymal fibroblastic cells migrating from the adjacent bone marrow space. The membrane structures, cytokine productions, and other biological characteristics are very similar among those fibroblastic cells derived from these two origins. These

cells were found to have a characteristic biological function; holding lymphocytes underneath and supporting the development and proliferation of these cells. This function named "pseudoemperipolesis" was originally found by Dr Wekerle (1980) in thymus cells of rats and mice, and those fibroblastic cells were named as nurse cells. We established the mesenchymal fibroblastic cell lines from synovial tissue and bone marrow cells in RA patients, and found the pseudoemperipolesis in these fibroblastic cells (nurse-like cells; NLC) just like nurse cells.

We isolated monocytes from the peripheral blood of healthy donors, and incubated with NLC from RA patients (RA-NLC). After 4 weeks of culture, TRAP- positive mononuclear cells with larger cytoplasm appeared. Monocytes cultured in medium alone died within 6 weeks. These TRAP- positive mononuclear cells differentiated into the multinucleated giant cells by incubating with some cytokines even in the absence of RA-NLC. These multinucleated giant cells showed the bone-resorbing activity by culturing on dentine slices. Considering that the significantly higher number of TRAP- positive mononuclear cells and the much more nucleated giant cells with higher bone-resorbing activity could be obtained from the iliac bone marrow of patients with more erosive disease group, RA-NLC could be considered to play important roles in highly activated bone destruction (including severe secondary osteoporosis) of RA patients.

## L22

No abstract

## L23

### Molecular events in cartilage formation and remodeling

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Cartilage extracellular matrix contains a major component of highly anionic proteoglycan contributing fixed charges creating and osmotic environment and a swelling pressure important for resisting pressure load. Another key element is a network of fibers with collagen 2 as the major constituent providing tensile properties and an ability to take up load.

In forming the cartilage matrix the cells produce the macromolecules that constitute the building blocks. These are assembled into the structures of the tissue outside of the cells in a number of specific interactions. An example is the fiber network where collagen molecules form fibrils by interactions where a variety of matrix molecules act as catalysts/chaperons or inhibitors.

Examples of molecules interacting with collagen are particularly found among the leucine rich repeat proteins (LRRP). These include decorin, fibromodulin, lumican and biglycan all with known capacity to bind collagens and influence fibrillogenesis in vitro. This binding occurs via the LRR-domain. Furthermore, the molecules have an additional functional domain, that in the case of decorin carries dermatan sulfate chains capable of interacting with other constituents in the matrix including other collagen fibers thereby crossbridging and creating a fibrillar network covering large parts of the tissue.

In the case of decorin, lumican and fibromodulin, mice with inactivated genes show alterations in collagen fibril assembly indicative of roles at different stages of the process. PRELP binds collagen via its repeat domain and heparan sulfate via a characteristic N-terminal extension. This includes binding heparan sulfate at the cell surface. Chondroadherin binds cells via their  $\alpha 2\beta 1$  integrin. The molecule can actually be isolated from cartilage bound to collagen 2 molecules after activation of endogenous proteinases.

COMP represents a different class of molecules with five identical subunits held together in their N-terminal end. The C-terminal end of each chain has a structure allowing tight and specific interactions with triple helical collagen. There are four sites along the collagen molecule each with a KD of 10<sup>-9</sup>. COMP in vitro has a marked effect in catalyzing the correct assembly of collagen fibers, while not binding to the completed fiber. Thus, the molecule act as a chaperon.

Interestingly COMP is upregulated in early phases of osteoarthritis, where a repair attempt of the damaged tissue is likely to be a component. The molecule or fragments thereof released to synovial fluid and blood, actually serves as an indicator of processes in the cartilage leading to its destruction.

In processes in cartilage remodeling, many of the constituents in the matrix are degraded and lost to surrounding body fluids. This degradation is likely to be a response to remodeling following material fatigue, altered load or growth. It may also occur as part of a pathological process. It is likely that it is coupled to attempts at repair laying down new matrix constituents to produce an adequately functioning matrix. In disease it is apparent that the imbalance between breakdown and adequate repair leads to progressive changes in cartilage composition characteristic for the various stages of the disease.

## L24

### The molecular mechanism of osteoclastogenesis: ODF/RANKL-dependent and independent pathways

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It is well established that osteoblasts and bone marrow stromal cells express osteoclast differentiation factor (ODF, also called RANKL) in response to several bone-resorbing factors to support osteoclast differentiation from their precursors. Osteoclast precursors which express RANK, a TNF receptor family member, recognize ODF/RANKL through cell-to-cell interaction with osteoblasts/stromal cells, and differentiate into osteoclasts in the presence of M-CSF. Osteoclastogenesis inhibitory factor (OCIF, also called OPG) acts as a decoy receptor for ODF/RANKL. ODF/RANKL is responsible for inducing not only differentiation, but also survival and activation of osteoclasts.

IL-1 and TNF $\alpha$  also play a major role in the pathogenesis of bone resorption induced by inflammation. IL-1 induced osteoclast differentiation by a classical ODF/RANKL-dependent mechanism, indicating that osteoblasts are essential for IL-1-induced osteoclast formation. In contrast, mouse TNF $\alpha$  strongly stimulated differentiation of M-CSF-dependent bone marrow macrophages (M-BMM $\phi$ ) into osteoclasts without any help of osteoblasts/stromal cells. Osteoclast formation by TNF $\alpha$  was inhibited by antibodies against TNF receptor type 1 and 2 (TNFR1 and TNFR2), but not by OPG/OCIF, indicating that differentiation of M-BMM $\phi$  into osteoclasts by TNF $\alpha$  occurs by a mechanism independent of the ODF/RANKL-RANK interaction. IL-1 failed to induce differentiation of M-BMM $\phi$  into osteoclasts.

More recently, we found that lipopolysaccharides (LPS)-induced bone loss did not occur in knockout mice of EP4, a subtype of PGE<sub>2</sub> receptor. This indicates that EP4 signals are involved in the LPS-induced bone resorption. LPS appeared to induce osteoclast formation by two different pathways: one is an ODF/RANK-independent pathway involving TNF $\alpha$ . LPS induces TNF $\alpha$  production through toll-like receptor 4 (TLR4) in macrophages, which in turn directly acts on osteoclast progenitors through TNFR1 and TNFR2 to induce osteoclast differentiation. In this pathway, osteoblasts did not appear to be

involved. The other pathway is the classical ODF/RANKL-dependent pathway. In the classical pathway, LPS induces PGE<sub>2</sub> production through TLR4 in osteoblasts and macrophages, which in turn induces ODF/RANKL through EP4 in osteoblasts. ODF then binds ODF receptor (RANK) in osteoclast progenitors by cell-cell contact, which stimulates osteoclast differentiation.

We conclude that osteoblasts/stromal cells are involved in not only physiological, but also pathological bone resorption via ODF/RANKL.

## L25

### Normal and pathological bone development controlled by the AP-1 transcription factor complex

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c-Fos is a key regulator of bone development, since transgenic mice expressing exogenous Fos develop bone tumors, whereas mice lacking c-Fos are osteopetrotic due to a differentiation block in bone resorbing osteoclasts. We are interested to study how c-Fos and its related protein Fra-1, which is c-Fos inducible, control osteoblast proliferation and osteoclast differentiation (1). We recently found that Fra-1 is an essential gene for mouse development (2) and transgenic mice overexpressing Fra-1 develop the bone disease osteosclerosis, which is due to increased bone formation (3). To test whether Fra-1 can substitute for c-Fos, we generated knock-in mice that express Fra in place of c-Fos. Fra-1 rescues c-Fos dependent functions in bone development which appeared to be gene dosage dependent (4). However, Fra-1 failed to substitute for c-Fos in inducing expression of target genes *in vitro*. We are using these systems to identify novel Fos target genes by microarrays and with the help of bone-specific conditional alleles of c-Fos and Fra-1, we are studying the molecular mechanisms how Fos proteins govern bone cell development and differentiation.

Since Fos proteins need Jun proteins to activate transcription, we investigated the function of c-Jun in bone cells using the *cre/loxP* system. Chondrocyte-specific inactivation using *col2A1-cre* transgenic mice results in severe scoliosis caused by failure of intervertebral disk formation and abnormal vertebral arch development, suggesting that c-jun is a novel regulator of sclerotomal differentiation.

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## P119

### Interphase fluorescence *in situ* hybridization analysis of fibroblast-like synoviocytes of patients with rheumatoid arthritis and osteoarthritis

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Synovial stromal cells (e.g. fibroblast-like synoviocytes, FLS) are thought to play an essential role in the pathogenesis of inflammatory joint diseases, in particular in the destructive aspects of rheumatoid arthritis (RA). Recent evidence indicates that chromosomal alterations have a profound impact on cellular behavior, even in non-transformed cells. We therefore investigated whether or not alterations in chromosome number occur in FLS of patients with RA and osteoarthritis (OA).

Synovial tissue was collected at the time of joint surgery from 21 patients with RA and 22 patients with OA. Synoviocytes were isolated by enzymatic dispersion. Interphase fluorescence *in situ* hybridization (FISH) analysis of freshly isolated synoviocytes and stimulated or unstimulated cultured FLS was performed using DNA probes specific for chromosome 17, 8, 11, 6, 7 centromeres and the p53- (17p13), c-myc- (8q24), and the retinoblastoma gene-1- (13q14) gene locus.

In all the patients studied, both RA and OA, concordant signal numbers with the probes recognizing chromosome 17, 8, 11 centromeres, 17p13, 8q24, and 13q14 were obtained (dual color FISH), indicating that allelic losses or gains of p53, c-myc, or the retinoblastoma gene-1 are not prevalent in fibroblast-like synoviocytes. Using  $\alpha$ -satellite DNA probes specific for chromosome 6 or 7, alterations in chromosome number were identified in synoviocytes derived from some patients with both RA and OA.

In fibroblast-like synoviocytes, alterations in chromosome number and subsequent selection of chromosomally altered cells may occur in the joints of patients and contribute to the perpetuation of synovitis.