Abstracts

Immune regulation

001 IL-7 MODULATES THE SUPPRESSIVE FUNCTION OF CD4+CD25HIGH REGULATORY T-CELLS

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Recently, IL-7 was shown to down-regulate CD4+CD25high regulatory T-cell (T-reg) function in vitro, both in animal models and human cells. We previously showed reduced circulating levels of IL-7 in rheumatoid arthritis (RA)[1] as well as reduced numbers of circulating T-reg in early, DMARD-naive RA patients.[2] Here we took advantage of a well characterised cohort of patients with RA whose disease was well controlled, and where we had demonstrated heterogeneous circulating levels of IL-7 (ranging from almost none to above normal levels,[1] to investigate the role of IL-7 on CD4+CD25high T-reg in vivo. Peripheral blood samples were taken from patients with RA whose disease was well controlled. Serum IL-7 levels were measured by ELISA and CD4+CD25high T-reg quantified by flow cytometry. Circulating levels of IL-7 positively correlated with the frequency of circulating T-reg ($n=47$, $R=+0.647$, $P<0.00001$). the proliferative history of CD4+CD25high T-reg was evaluated by real-time PCR quantification of TRECs (T cell receptor excision circles) in CD4+ T-cells following cell sorting based on CD25 expression levels. Higher frequency of CD4+CD25high T-reg in relation with IL-7 appeared to result from an increased production of these cells by the thymus and not from the homeostatic proliferation of T-reg in response to higher levels of circulating IL-7. Thymidine incorporation assays were further used to assess the response of CD4+CD25high T-reg to IL-7 stimulation, and also their ability to suppress the proliferation of CD4+CD25- T-cells in response to phytohaemagglutinin (PHA) in co-culture. The ability of T-reg to suppress the proliferation of CD4+CD25- T-cells in vitro was directly associated with their prior exposure to IL-7 in vivo ($n=8$, $R=+0.786$, $P=0.021$). However, it is well known that IL-7 is highly expressed in the synovium. Synovial fluid was obtained from RA and OA patients following clinical indication for aspiration and IL-7 measured. We observed higher IL-7 levels in the synovial fluid of RA patients ($n=9$, $13.7+/-8.7$ pg/ml) compared to OA ($n=8$, $6.67+/-2.7$ pg/ml, $P=0.001$). The suppression of CD4+CD25- T-cell proliferation by T-reg was abolished when the co-cultures were done in the presence of IL-7, as reported by others. Our data therefore suggest that IL-7 has a role in regulating T-reg number and function in the periphery. Circulating IL-7 levels are low in active rheumatoid arthritis,[1] and this may be a contributory factor to the reduced size and suppressor function of the circulating T-reg population in this disease. In addition, high IL-7 in the joint will further contribute to the reduced ability of T-reg to perform immune regulation locally.

1. **Ponchel**, *et al. AR&T* 2005;**7**:80–92.
2. **Lawson**, *et al. BSR*, 2003, Manchester.

002 B CELLS ARE POTENT STIMULATORS OF ENDOSTEAL BONE FORMATION IN INFLAMMATORY ARTHRITIS

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Introduction: Inflammatory arthritis can destroy the entire cortical bone barrier, which leads to exposure of bone marrow to synovial tissue followed by generation of bone marrow infiltrates. These mononuclear cell infiltrates, which separate the bone marrow from synovial tissue, mainly consists of B cells. The observation in clinical studies, for example treatment of RA patients with rituximab, indicates that depletion of B cells from RA patients results in a significant therapeutic effect, suggesting that B cells can play an important role in disease pathogenesis. However the exact role of the B cells of the disease process has not been established yet and may extend to mechanism independent from autoantibody production.
Methods: To analyse the role of the B-cells in the pathology of inflammatory arthritis, hTNFtg mice, an established animal model for arthritis, were interbred with Brutons tyrosine kinase (Btk) knock-out mice, which show a developmental defect of B cells. Offspring of four different genotypes (WT, Btk-/-, hTNFtg and Btk-/-hTNFtg) were analysed for clinical and histological signs of arthritis. Areas of inflammation were evaluated with H&E-stained sections, bone erosion was quantificated by TRAP-stained sections of hind paws. Osteoid formation by osteoblasts was visualised on Movat-stained paraffin sections of paws.
Results: There was no destinction in the clinical course of arthritis between hTNFtg and Btk-/-hTNFtg mice. Histological examination of inflammation and bone erosion in the hind paws showed no difference in hTNFtg and Btk-/-hTNFtg mice. Btk-/- hTNFtg mice developed bone marrow infiltrates with smaller extend and a reduced number of B cells. The number of osteoblasts at endosteal sides next to bone marrow infiltrates was reduced in Btk-/-hTNFtg mice compared to hTNFtg mice. This leads to higher extend of inflammation in the vicinity of these bone marrow infiltrates in Btk-/-hTNFtg mice.
Conclusion: These results indicate that bone formation at endosteal regions next to bone marrow infiltrates is driven by B cells. This leads to the assumption that B cell attempt to counterregulate the process of bone resorption by recruitment of osteoblasts.

003 RELAPSE IN RA PATIENTS IN CLINICAL REMISSION CAN BE PREDICTED USING T-CELL DIFFERENTIATION MARKERS

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We previously demonstrated abnormal T-cell differentiation with a particular subset of CD4+T-cells (CD45RBbright CD45RA+ CD45RO+/- CD62L- called IRCs) in early and more advanced RA in direct relationship with inflammation.[1] Flow cytometry was used to assess T-cell differentiation pattern in 42 patients in remission and the frequency of IRCs was calculated. IRCs persisted in the absence of systemic evidence of inflammation as well as clinical sign of synovitis (clinical remission). Here we investigate the clinical significance of this observation and found it to be predictive for relapse. The molecular mechanism of this observation is investigated in an associated poster. Patients were defined as ''in remission'' when they had controlled disease for at least 6 months, with no change of activity, CRP below 12, no swollen or tender joints and were on stable treatment (with or without therapy). A complete set of clinical data was collected on these patients. Statistical analysis was used to seek clinical and laboratory correlate to differentiation abnormalities. The persistence of was not associated with any demographic or clinical parameters such as sex, age, disease duration prior to remission, duration of remission, CRP (between 0 and 12 mg/L), RF, ACR criteria for RA or remission, NSAID, nodules, current or prior drug history, age at onset of disease, smoking or patient reported family history of arthritis. We also could not associate the persistence of IRCs with a cumulative history of inflammation. Relapse was defined as a change in disease activity (increased DAS28), new symptoms sustained for at least 3 months, requiring or not a change in treatment. Relapse occurred in 20 out of 42 patients within 18 months of followup and was associated with a higher frequency of IRCs at base line in the blood ($OD=6.40$, $P=0.009$). We therefore proceeded to a complete analysis of factors that could predict relapse. Only RF positivity ($OD=3.9$, $P=0.041$) and possibly having nodules ($OD=3.4$, $P=0.121$) were also associated with relapse. Using a regression analysis revealed that the frequency of IRCs was highly significant in predicting relapse, being correct in 78% of cases ($OD=7.57$, $P=0.010$). Neither the presence of RF ($OD=4.85$, $P=0.046$) or nodules (NS) improved this model. These results suggest that the persistence of IRCs in the circulation of patients in clinical remission could be used to predict relapse and modulate the need for monitoring of these patients. The molecular mechanism by which these cells persist in the circulation and could be responsible for initiating relapse is described in an associated poster.

1. **Ponchel**, *et al. Blood* 2002;**100**:4550–6.

004 MOLECULAR MECHANISM OF THE PERSISTENCE OF ABNORMAL T-CELLS IN RA PATIENTS IN CLINICAL REMISSION: INFLAMMATION RELATED CELLS

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We previously demonstrated abnormal T-cell differentiation in early and more advanced RA.[1] We observed a subset of CD4+ T cells (CD45RBbright CD45RA+ CD45RO+/− CD62L-) associated with inflammation (called IRCs). However, in the absence of systemic

evidence of inflammation as well as clinical signs of synovitis (clinical remission), these IRCs persisted. We investigated first the clinical significance of this observation (associated poster) and second its molecular mechanism. Patients were defined as ''in remission'' when they had controlled disease for at least 6 months, with no change of activity, CRP below 12, no swollen or tender joints and on stable treatment. Statistical analysis was used to seek clinical and laboratory correlate to differentiation abnormalities. T-cell differentiation patterns were analysed by flowcytometry and the frequency of IRCs was calculated. 42 remission patients were compared with 30 active RA and 30 controls. The abnormal differentiation patterns observed in active disease in direct relation with inflammation, were maintained in remission. This was not associated with any demographic or clinical parameters. We also used proliferation assay (n = 8) to determine that the hyper-proliferation associated with IRCs was lost in remission and therefore appears to have been driven by inflammation. The expression of chemokine receptors (CXCR3, CXCR4) was measured by flowcytometry (controls n = 10, RA n = 10, remission n = 20). The expression of the chemokine receptors CXCR3 and CXCR4 on IRCs in remission was significantly lower compared to active disease ($P<0.01$) suggesting that a change in trafficking between the peripheral blood and the synovium may be responsible for the presence of IRC in the circulation. ELISA were used to measure circulating TNF, TGF and IL-2 (controls n = 10, RA n = 10, remission n = 20). There was no relationship between the frequency of IRCs and any circulating cytokines. Finally, real time PCR was used to quantify the expression of Bcl-2 and Bax (controls n = 10, RA n = 10, remission n = 20). Considering that we had to use PBMCs, there was a significant drop in Bax expression in patients in remission compared to active disease ($P<0.0001$), and a significant inverse correlation between Bax expression and IRC frequency ($R=-0.830$, $P=0.021$). These results suggest that the persistence of IRCs in the circulation of patients in remission can be due to a change in trafficking from the synovim to the circulation. IRCs may then survive in remission due to their low levels of Bax expression possibly acquired during their stay in the synovium. The survival of IRC in remission certainly confirms the important role of these cells in RA, as circulating precursors of pathogenic cells aiming for the joint under inflammatory conditions and may explain why patients with higher frequency of IRCs in remission have a higher risk of relapsing (see associated poster).

1. **Ponchel**, *et al. Blood* 2002;**100**:4550–6.

005 MANNOSE BINDING LECTIN, A NEW CANDIDATE MOLECULE TO EXPLAIN AMELIORATION OF RHEUMATOID ARTHRITIS DURING PREGNANCY?

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Introduction: Rheumatoid arthritis (RA) ameliorates during pregnancy. The mechanism underlying this phenomenon is unknown.

Mannose Binding Lectin (MBL) and L-ficolin activate the lectin complement pathway by binding to MBL-associated serine proteases (to be determined as MBL complex-activity). Finally the C5b-C9 complex will be formed, which is the endproduct of complement activation (to be determined as lectin route activity).

The MBL serum concentration is mainly determined by its genotypes (AA high producers, A0/00 low producers). In RA high levels of MBL are associated with less active disease and lower autoantibody levels.

Increased levels of MBL during pregnancy could therefore be an explanation for the pregnancy-induced remission of RA.

Aim: To determine to what extent L-ficolin and MBL serum concentrations and its subsequent steps in complement activation are influenced by pregnancy. Moreover to investigate the role of the MBL-genotype herein.

Materials and Methods: MBL and L-ficolin concentrations and the various steps of complement activation were determined by ELISA in sera from healthy women (n = 32) during every trimester of their pregnancies and three times postpartum. Genotyping for MBL was performed by PCR.

Results: MBL concentrations rise during pregnancy (mean 1.6 times (range 0.6–5.4), in all genotypes studied ($p<0.003$). This increase is already present in the first trimester and does not rise much during pregnancy. Directly postpartum (6 weeks) MBL concentrations drop sharply (mean 0.6 times (range 0.1–0.9) and return to baseline level in about three to six months ($p<0.0001$). Changes in MBL concentrations were directly reflected by changes in MBL complex-activity and lectin route activity. R between the all parameters >0.93 at all time points ($p<0.01$).

In individuals with the AA genotype the pregnancy-related increase in MBL serum levels, complex-activity and lectin route activity is higher than in the other genotypes, both in absolute and relative values. The postpartum drop occurs equally in all genotypes.

L-ficolin serum concentrations do not show comparable variations during pregnancy or postpartum.

Conclusion: MBL concentration, complex-activity and lectin route activity rise significantly during pregnancy, drop sharply directly postpartum and return to baseline after about 3 to 6 months. The positive correlation between MBL concentration and MBL complex activity indicates that the rise in MBL concentration is of functional importance.

The results obtained in this study justify further research in elucidating a potential role for MBL in pregnancy-induced amelioration of RA. This will be done in the nationwide prospective cohort study on RA and pregnancy that was started in the Netherlands in 2002.

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006 IL-13 SKEWS THE BALANCE BETWEEN THE ACTIVATING AND INHIBITORY FC GAMMA RECEPTORS (FCGRS) TOWARDS THE INHIBITORY SUBTYPE (FCGRIIB) ON DENDRITIC CELLS

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Introduction: Fc gamma receptors (FcgRs) and Toll-like receptors (TLRs) control the function of dendritic cells (DCs). The balance between the activating FcgRI, IIa and IIIa and the inhibitory FcgRIIb determines the type of immune response upon IgG binding. Whereas the stimulatory effect of TLRs on DC activation is well established it is still largely unknown how TLR mediated responses are inhibited.

Aim: Here we aimed to investigate the influence of the anti-inflammatory cytokines IL-4, IL-13 and IL-10, on the balance between activating and inhibiting FcgR subtypes. In addition, we studied whether FcgRIIb triggering inhibits TLR mediated signaling.

Materials and Methods: DCs were derived from peripheral blood mononuclear cells from healthy controls (N = 15). Adherent monocytes were cultured with GM-CSF and with IL-4 or IL-13 and either with or without IL-10 for 6 days. The expression of the FcgRs was assessed using flowcytometry. The secretion of TNF-a, IL-6 and IL-10 was measured after stimulation with LPS (TLR4 pathway) alone or LPS plus immune complexes (FcgR pathway) on DCs cultured in the presence of IL-4 or IL-13 and either with or without IL-10, using Luminex technology.

Results: Compared with IL-4 DCs, IL-13 DCs displayed a high expression level of FcgRIIb (66 ± 8 vs. 92 ± 12, $P=0.003$ (mean MFI $\pm$ SEM) resulting in an FcgR balance profoundly skewed towards the inhibitory FcgRIIb. The addition of IL-10 to either IL-4 or IL-13 increased both FcgRIIa and IIb, resulting in a less skewed balance to FcgRIIb. Of note, IL-13 does not sort any effect on FcgRI or FcgRIII, while IL-10 increases the expression of FcgRIIIa and has no effect on FcgRI. Only IL-13 DC inhibited the production of inflammatory mediators upon co-stimulation with LPS and IC compared to stimulation with LPS alone. The expression of FcgRIIb on IL-13 DCs correlated strongly with the inhibition of TNF-a secretion upon IC mediated triggering ($R2=0,84$; $p=0,002$). In contrast, LPS activated IL-4 DCs and DCs differentiated with combinations of IL-10/IL-4 and IL-10/IL-13 increased the production of TNF-a, IL-6 and IL-10 upon IC co-stimulation demonstrating that alterations in the FcgR balance have clear functional consequences.

Conclusion: In conclusion, we here show that IL-13 selectively increases the expression of FcgRIIb resulting in the inhibition of TLR mediated DC activation via immune complexes. Further research to elucidate the functional consequences for DC function is warranted and might underscore the rational to target the inhibitory FcgRIIb to combat autoimmunity.

007 VITAMIN D INHIBITS IL-17 PRODUCTION AND STIMULATES IL-4 PRODUCTION BY PERIPHERAL BLOOD MONONULCEAR CELLS IN EARLY ARTHRITIS PATIENTS

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Background: T cell interleukin (IL)-17 is a proinflammatory cytokine in rheumatoid arthritis (RA) and is a potent inducer of inflammatory cytokines such as IL-1, tumour necrosis factor alpha, and RANKL, stimulating osteoclastogenesis and bone resorption in RA. IL-4 is an anti-inflammatory cytokine that inhibits formation of osteoclasts and may stimulate bone formation. Several studies have shown that

1,25-dihydroxyvitamin D3 (vitamin D) has immunomodulatory effects and vitamin D suppletion can prevent autoimmune collagen arthritis.
Objective: To examine the effect of vitamin D on pro-inflammatory (IL-17) and anti-inflammatory (IL-4) cytokines production by peripheral blood mononuclear cells (PBMC) and to assess the association with early arthritis.
Methods: 8 early arthritis patients and 8 healthy controls (2 men and 6 women per group, aged 30–66 years and 31–67 years, respectively) were included. PBMC from early arthritis patients and healthy volunteers were isolated. PBMC were stimulated for 72 hours with antiCD3/antiCD28 in the absence and presence of different concentrations of vitamin D. Cytokines were measured in the supernatant by specific ELISAs.
Results: In supernatantia of unstimulated PBMC IL-17, IL-4 and interferon gamma (IFNγ) production were below detection level.

AntiCD3/antiCD28 stimulated PBMCs showed a significant increase in IL-17 production (800 pg/ml in early arthritis patients and healthy controls). Co-incubation with vitamin D significantly suppressed this IL-17 production dose-dependently (maximal inhibition about 60%). AntiCD3/antiCD28 stimulated IFNγ production (8000 pg/ml in both groups) was completely blocked by vitamin D. Interestingly, incubation with vitamin D resulted in a twofold increase in IL-4 production in all participants (55 to 110 pg/ml). The ratio of IL-17/IL-4 production by antiCD3/antiCD28 stimulated PBMCs was dose-dependently decreased by vitamin D: ratio of 18 after antiCD3/antiCD28 incubation, and 7 and 3 when coincubated with 10-9 M and 10-7 M vitamin D, respectively.
Conclusion: These data show the potential of vitamin D to increase the regulatory/anti-inflammatory cytokine IL-4 and to suppress the pro-inflammatory cytokine IL-17 by PBMC from early arthritis patients. These data suggest a beneficial effect of vitamin D on the anti-inflammatory/pro-inflammatory T cell cytokine balance in early arthritis and thereby potentially may have bone sparing effects in early arthritis.

008 IMMUNOMICS IN INFLAMMATORY RHEUMATIC DISEASES

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Autoimmune diseases such as rheumatoid arthritis (RA) are characterised by autoantibodies to different autoantigenic proteins. Using proteomic 2D immunoblots, we identified a new 40 kDa autoantigen – hnRNP A3 – from HeLa nuclear extracts, which is frequently (30%) detected by RA. Moreover, we used a set of protein arrays of about 50 000 proteins derived from a human foetal brain cDNA expression library for screening with patient sera. Additionally, we utilised a human foetal brain cDNA library in a robot-based T7 phage display screening system with RA patient sera. To determine the diversity of the enriched library, we amplified the cDNA inserts and hybridised them onto the custom-made human ENSEMBL cDNA array. By these methods, over 80 clones were identified to bind patient immunoglobulins. Moreover, nine clones showed only IgA-specific reactivity. We have now evaluated two different clones thoroughly: the carboxyl-terminal half of the nucleolar phosphoprotein p130 (NOPP 130) and a clone representing a 41-amino-acid mimetic peptide. Affinity purified antibodies from this peptide showed a nuclear membrane staining and to react with a 90 kD protein. The reactivity to the remaining proteins is still undergoing thorough investigation. Applying state-of-the-art proteomic techniques such as protein array and phage display, we have succeeded identifying more than 80 potentially autoantigenic marker molecules, with which we have characterised a subset for RA specificity by screening with large numbers of patient and control sera. Moreover, this autoimmune autoantigen profile is currently compared to expression profiles of these autoantigens in synovial membrane from RA patients and controls. This is the first time that expression profiles and autoimmune profiles are compared using this novel approach high throughput.

009 DECREASED PD-1 BUT NOT CTLA-4 EXPRESSION IN PERIPHERAL BLOOD T CELLS OF PATIENTS WITH INACTIVE SLE: IMPLICATIONS FOR PERIPHERAL TOLERANCE

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Background: Programmed death-1 (PD-1) is a newly described coinhibitory receptor of the B7/CD28 superfamily that is expressed on activated T- and B-lymphocytes. Engagement of PD-1 by its specific ligands (PD-L1 and PD-L2) results in decreased activation of lymphocytes, and PD-1$^{-/-}$ mice develop autoimmune disease, both indicating an important role for PD-1 in peripheral tolerance. Single-nucleotide polymorphisms in the human PD-1 gene have been associated with increased risk for systemic lupus erythematotus (SLE) and lupus nephritis.
Aim: We sought to examine the baseline, kinetics of expression and the function of PD-1 on peripheral blood mononuclear cells (PBMCs) of patients with SLE.
Materials and Methods: PBMCs were isolated from patients with SLE (n = 38), rheumatoid arthritis or other inflammatory disease (n = 26), and healthy donors (n = 20). SLE disease activity was determined by the SLE Disease Activity Index (SLEDAI), and active disease was defined as SLEDAI $\geq$8. Expression of PD-1, PD-1 ligand 1 (PD-L1), and CTLA-4 (intracellular) was assessed by flow cytometry. PBMCs were stimulated ex vivo by PMA/ionomycin to examine kinetics of PD-1 expression.
Results: SLE patients with inactive disease had decreased percentage of PD1$^+$CD3$^+$ peripheral blood lymphocytes (mean $\pm$ S.E.M., 0.5$\pm$0.1%, n = 16), compared to patients with active disease (1.1$\pm$0.3%, n = 22, $p<0.05$), healthy donors (0.8$\pm$0.1%) or disease controls (1.1$\pm$0.2%). No difference was found in percentage of PD1$^+$CD19$^+$ B cells (1.8$\pm$0.3% in inactive vs. 2.1$\pm$0.4% in active disease, $p>0.05$). The proportion of PD1$^+$CD4$^+$ cells was also decreased in SLE patients with inactive (0.6$\pm$0.1%, n = 4) compared to those with active disease (2.2$\pm$0.4%, n = 8, $p<0.05$). In contrast, no difference was found in CTLA-4$^+$CD4$^+$ cells in patients with inactive vs active disease (22.6$\pm$5.1% vs. 18.3$\pm$5.5%, respectively, $p>0.05$). Preliminary results demonstrate reduced percentages of PD1$^+$ CD4$^+$CD25$^+$ and PD1$^+$ CD4$^+$CD69$^+$ peripheral blood lymphocytes in patients with inactive compared to active disease (0.6$\pm$0.2% vs 2.3$\pm$1.6%, $p<0.05$, and 2.0$\pm$1.3% vs 3.8$\pm$1.9, p = 0.05, respectively). Following stimulation with PMA/ionomycin for 48 hours, patients with inactive disease have reduced percentage of PD1$^+$CD3$^+$ T lymphocytes compared to patients with active disease (14.6$\pm$2.5% vs. 25.6$\pm$4.7%, $p<0.05$). Experiments are underway to investigate possible differences in function of PD-1 among patients with SLE, using PD-L1.Fc protein to crosslink PD-1.
Conclusions: SLE patients with inactive disease had reduced percentage PD1$^+$ but not CTLA4$^+$ peripheral blood T lymphocytes compared to patients with active disease, both at basal level and following stimulation with PMA/ionomycin. The reduced expression of PD-1 on PBMCs of these patients might contribute to dysregulated peripheral tolerance.

010 FOXP3 TRANSCRIPTION FACTOR IS NOT CONFINED TO REGULATORY T (TREG) CELLS: HUMAN EPITHELIAL CELLS EXPRESS FOXP3 MRNA

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Introduction: FOXP3 is a transcription factor that has been implicated in both the development and function of Treg cells. Treg cells comprise a subset of CD4+ T cells that are characterized by the high constitutive expression of CD25 molecule and their ability to suppress the proliferation and function of effector T lymphocytes, as well as other immunocytes, thus playing a pivotal role in immune regulation. Until recently, the study of Treg cells had been hampered by the lack of a specific marker, but the identification of FOXP3 provided the only up to date exclusive Treg marker with a documented functional role, specifically in the mouse model.

In humans, FOXP3 has also been documented as a Treg marker. In this study we aimed to investigate if the expression of FOXP3 mRNA is exclusive to Treg cells in humans. Hence, FOXP3 mRNA expression was analyzed in ''non-immune cells'' such as the human non-neoplastic salivary gland epithelial cell (SGEC) lines, the HeLa (cervical adenocarcinoma), the PC3 (prostate) and the MDA (mammary) neoplastic epithelial cell lines, as well as in HUVEC (human umbilical vein endothelial cells).
Materials and Methods: The expression of FOXP3 mRNA was investigated by RT-PCR specific for the entire coding region. The PCR products were identified by automated sequencing. FOXP3 mRNA expression levels were evaluated by real-time PCR. Each sample was analysed in triplicates and the relative quantification was performed by ΔCTs analysis, using the human HPRT as a reference gene and magnetic bead-purified CD4+CD25+ T cells as the calibrator sample.
Results: All the epithelial cell lines tested were found to express two FOXP3-PCR products of approximately 1300 and 1200 bp that correspond to the full-length FOXP3 mRNA and to a previously reported FOXP3 alternate transcript, respectively. None of the FOXP3 transcripts was detected in HUVEC. Real-time PCR analysis revealed that epithelial cells express significantly lower levels of FOXP3 mRNA compared to CD4+CD25+ T cells. More specifically, FOXP3 mRNA levels (expressed as a percentage relative to the expression of CD4+CD25+ cells;100% by

default) were found to be 0.21% in SGEC, 0.05% in HeLa, 0.32% in PC3, 0.07% in MDA.
Conclusion: These findings support that FOXP3 mRNA expression is not confined to T cells in humans. Epithelial cells also express low levels of FOXP3 mRNA. The significance of this expression, as well as the expression of FOXP3 protein by epithelial cells needs to be further investigated.

011 ANALYSIS OF THE INTERACTION OF CD4+CD25+ REGULATORY T CELLS WITH DENDRITIC CELLS

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CD4+CD25+ regulatory T cells (Treg) mediated suppression of effector T cell proliferation has been shown to be cell-cell contact dependent. Whether suppression also requires cognate interaction with antigen presenting cells (APC), especially with dendritic cells (DC), however, remains controversial.

We therefore analysed the interaction of isolated human peripheral blood CD4+CD25+ Treg with CD1c+ myeloid DC in vitro.

Cell aggregate formation was observed in cultures of Treg with DC after 6h. Aggregate formation occurred upon pre-activation of Treg with anti-CD3 monoclonal antibody (mAb) and to a lesser extent upon activation of DC with granulomonocyte colony stimulating factor (GM-CSF). Almost no aggregates were observed in co-cultures of resting Treg with DC.

The composition of cell aggregates was further analysed by laser scanning microscopy after staining of different cell populations with distinct membrane bound fluorescence dyes. This confirmed the formation of aggregates between Treg and DC. Inhibition experiments with mAb against several adhesion molecules in addition revealed a role of LFA-3, ICAM-1 and ICAM-3 molecules for aggregate formation.

Phenotypic analysis of DC after 12h co-culture with either T reg or CD4+CD25- effector T cells by flow cytometry (FACS) revealed an increased expression of activation-associated molecules like CD40, CD80, CD83, CD86 and HLA-DR in the presence of Treg or effector T cells, especially upon pre-activation of T cells with anti-CD3 mAb.

Ongoing experiments aim to determine a particular effect of Treg-DC interactions on the functional capacity of DC and/or Treg.

So far, our data demonstrate a strong tendency of activated Treg to interact with DC. This interaction might be required for Treg and/or DC function and be involved in the regulation of immunity and tolerance.

012 IMMATURE DENDRITIC CELLS SUPPRESS ARTHRITIS BY IN VIVO EXPANSION OF CD49B+ REGULATORY T CELLS

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Objective: Dendritic cells (DCs) are specialised antigen presenting cells with an important role in the initiation and regulation of immune responses. Immature DCs (iDCs) can mediate tolerance in the absence of maturation/inflammatory stimuli, presumably by the induction of regulatory T cells. Here, we explored the effects of the repetitive injection of iDCs on the development of collagen-induced arthritis (CIA) and investigate the cellular mechanism responsible of the observed effect.
Methods: DBA/1 mice were repetitively injected with PBS or 5×105 bovin collagen type II (bCII)-unloaded or -loaded iDCs, or bCII-pulsed TNF-DCs at days −7, −5 and −3 prior the immunisation with bCII in complete freund adjuvant. Mice were boosted on day 21 and clinical features of arthritis were determined untill day 50. Potential regulatory population were investigated after repetitive injections of the DCs. An expansion of the TCRb+CD49b+cells was observed in the mononuclear cells from the liver and in the spleen of the various DC-injected groups. These TCRb+CD49b+cells were sorted by FACS and 6×104 purified cells were injected intravenously to age-matched male DBA/1 mice one day before arthritis induction.
Results: Our results show that repetitive injection of unloaded immature dendritic cells protects significantly the animals as 70% of the mice did not develop clinical signs of the disease. The DCs injection was associated with an expansion of TCRb+CD49b+cells in the liver and spleen. In adoptive transfer experiment, only TCRb+CD49b+cells purified from the iDCs vaccinated mice protected efficiently mice from arthritis. This therapeutic effect was associated with a reduced collagen type II-specific antibody response induced after immunisation, as well as an enhanced IL10 secretion by draining lymph node cells.
Conclusion: Together these results show that iDCs can expand and activate a new regulatory population of CD49b+ T cells with high immunosuppressive potential able to mediate protection against a systemic autoimmune disease.

013 TOLL-LIKE RECEPTORS ON SENESCENT CD4+ T-CELLS: A NEW CONCEPT FOR CHRONIC ACTIVATION IN IMMUNE MEDIATED DISEASES

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Background: Toll like receptors (TLRs) allow the innate immune system the recognition of bacteria, viruses, fungi and even host material. Senescent, proinflammatory T-cells lacking the co-stimulatory molecule CD28, accumulate in chronic immune mediated disease and require alternative pathways for activation.
Objectives: To assess the expression and function of TLRs on CD28null T-helper cells in chronic immune mediated disease.
Methods: RT-PCR was conducted for mRNA expression of TLRs in CD4+CD28- and CD4+CD28+ T-cells. FACS analyses were performed for surface expression of CD14 and TLRs as well as for intracellular perforin and interferon-g production. In vitro assays were performed for (up-)regulation of TLRs by TNF-a and lipopolysaccharide mediated stimulation of TLR4 expressing T-cells.
Results: CD4+CD28null and CD8+CD28null T-cells are enriched in ankylosing spondylitis (AS), rheumatoid arthritis, psoriatic arthritis and polymyalgia rheumatica/giant cell arteritis compared to healthy controls ($p<0.01$ each). CD4+CD28- T-cells express mRNA for TLR1-10, whereas their CD28+ counterpart express mRNA only for TLR1, 2 and 9 at low levels. CD4+CD28null T-cells show higher levels of TLR2 and TLR4 on their surface compared to CD4+CD28+ or CD8+ T-cells ($p<0.05$ for each). TNF-a up-regulates TLR2 and TLR4 on CD4+CD28null T-cells in vitro and TNF-a blocking therapy decreases TLR2 and TLR4 expression in AS patients in vivo. Upon stimulation with LPS, CD4+CD28null T-cells produce perforin and interferon-g which is abrogated in the presence of TLR4- and CD14 blocking antibodies. Membrane-bound CD14 has been detected on the surface of CD4+CD28null T-cells and soluble CD14 in sera from AS patients.
Conclusion: These results demonstrate an alternative, TLR4 dependent mechanism of activation of CD4+CD28null T-cells in immune-mediated diseases, possibly responsible for chronic activation of these pro-inflammatory cells. These findings further support the hypothesis that CD4+CD28null T-cells represent an immunological link between the adaptive and the innate immune system.

014 NOVEL ACTIVATION MECHANISMS OF CD4 AND CD8 T CELLS IN CELL CONTACT MEDIATED MACROPHAGE DEPENDENT INFLAMMATION

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Background: T cell:macrophage cell - cell interactions are critical in perpetuating chronic synovitis in RA. CD4+ T cells isolated directly from RA synovial fluid (SF) or cytokine/mitogen activated CD4+ T cells induce TNF-α production by monocytes via a cell contact -mediated mechanism. Until now the role of CD8+ cells in this process has not been explored. Similarly the role of TLR ligands in driving such responses is unknown.
Aim: To examine whether CD8+ cells isolated from RA SF, like their CD4+ counterparts, have an inherent ability to drive monocyte activation. To investigate the potential role of TLR ligands in T cell-contact mediated stimulation of monocytes.
Methods: CD8+/CD4+ cells were isolated by immunomagnetic positive selection from RA synovial fluid and paraformaldehyde fixed without prior stimulation. CD8+ & CD4+ cells from healthy volunteer peripheral blood (PB) were activated by PHA, IL-15, anti-CD3/BLP, anti-CD3/Poly I:C or the cytokine cocktail (IL-6, IL-15 & TNFα) for 3/6 days, respectively. Fixed activated T-cells were then co-cultured with autologous CD14+ cells. Secreted cytokine production by monocytes was measured by ELISA/Luminex after 48 hours. The purity of isolated CD8+/CD4+ populations was assessed by FACS analysis was >92%. T cell markers subsequent to anti-CD3/TLR activation were assessed by FACS analysis.
Results: CD8+ cells isolated directly from RA SF induced autologous monocyte TNF-α secretion in the absence of prior in vitro activation. Levels of monocyte cytokine production stimulated by CD8+ cells were comparable to those induced by their CD4+ counterparts. In addition, mitogen, IL-15 or cytokine cocktail activated PB CD8+ cells induced TNF-α production by monocytes in a cell-contact dependent manner.

FACS analysis of BLP activated CD4+ T cells demonstrated enhanced expression of CD69 and ICAM-1. Moreover, CD4+ T cells activated by

the TLR 2 ligand BLP, but not TLR3 ligand Poly I:C, were capable of inducing monocyte TNF-α production.
Conclusion: The ability of CD8+ cells to stimulate monocyte inflammatory cytokine production via a contact - dependent mechanism provides an additional means by which T cell subsets activated within the local cytokine milieu in RA may contribute to the ongoing disease process. TLR activation of T cells provides a novel mechanism by which innate immunity may influence the adaptive immune response in RA.

015 ANTIBODIES AGAINST JUNCTIONAL ADHESION MOLECULE (JAM)-C DECREASE THE SEVERITY OF MURINE ANTIGEN INDUCED ARTHRITIS

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Junctional adhesion molecule-C (JAM-C) was described as an adhesion molecule localised at interendothelial contacts and as an integrin ligand mediating interactions between vascular cells and leukocytes, particularly during inflammatory cell recruitment. The aim of this study was to investigate the role of JAM-C in a model of antigen-induced arthritis (AIA).

JAM-C expression was studied by immunohistochemistry using a polyclonal antibody against murine JAM-C. AIA was induced by intra-articular injection of methylated bovine serum albumin (mBSA) into the knee joint of mBSA-immunised mice. Monoclonal anti-JAM-C (H36 or H33), or isotype matched control antibodies were injected (150 μg/mouse, i.p.) 1h before induction of AIA. Mice were sacrificed 4 or 8 days after induction of arthritis, the latter group receiving a second injection of antibodies on day 4. Arthritis was assessed by measuring 99mTechnetium (Tc) uptake in the knees and by histological scoring of knee joint sections. Lymphocyte and macrophage infiltration into the synovium was detected by immunohistochemistry using anti-CD3 and anti-MAC-2 antibodies. Serum amyloid A (SAA), anti-BSA antibody and interferon-gamma levels were measured by ELISA. Spleen cell proliferation was assessed by measuring 3H-thymidine incorporation.

JAM-C was highly expressed by synoviocytes of the lining layer and by some vessels in AIA synovial tissues. Treatment of mice with H36 significantly decreased the severity of arthritis, as quantified by Tc uptake on day 3. Histology showed lower inflammation in knees of H36 treated mice as compared to controls, lymphocyte and macrophage infiltration being similarly reduced. Serum SAA levels were maximal on day 4 and significantly decreased in H36 treated mice. Total anti-BSA antibodies and anti-BSA IgG2a were similar in H36 treated and control mice, while anti-BSA IgG1 were increased on day 4 in the H36 group. The ex-vivo proliferative response of spleen cells to concanavalin A was reduced in H36 treated mice, as was interferon-gamma production. Treatment of mice with H33 similarly decreased the T cell response, but neither modified anti-BSA IgG1 levels, nor reduced arthritis and serum SAA. The anti-arthritic effect of H36 can thus not be explained solely by changes in the T cell response, but appears to be related to additional anti-inflammatory effects of this antibody.

Treatment of mice with the monoclonal anti-JAM-C antibody H36 significantly reduced the severity of AIA, suggesting that JAM-C may represent a new therapeutic target in arthritis. The mechanisms underlying this effect are currently under investigation.

016 LONG TERM IFN-γ DEPENDENT EFFICACY OF ACTIVATED VALPHA14I NATURAL KILLER T CELLS IN THE PREVENTION OF COLLAGEN INDUCED ARTHRITIS

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Valpha14i Natural Killer T (iNKT) cells represent a unique immuno-regulatory T lymphocyte subset recognising glycolipid antigens presented by the major histocompatibility complex (MHC) class I-like molecule CD1d. Both the exogenous glycosfingolipids α-galactosylceramide (α-GalCer) and the C-glycoside analogue, α-C-galactosylceramide (α-C-GalCer), have been shown to be potent activators of iNKT cells, rapidly inducing large amounts of cytokines upon injection in mice. Whereas α-GalCer induces both IFN-γ and IL-4, α-C-GalCer, by contrast, induces prolonged production of IL-12 and IFN-γ and decreased production of IL-4, leading to a Th1 type immune deviation. We administrated these antigens to DBA/1 mice during the early induction phase of collagen-induced arthritis (CIA) and demonstrated potent therapeutic efficacy by a single administration of these antigens as monitored by clinical arthritis scoring and histopathology. Synovial mRNA levels of IL-1β and IL-6 in knee joints as well as systemic anti-type II collagen levels were found to be downregulated after treatment with either of the CD1d antigens. Interestingly, in vivo neutralisation of IFN-γ release induced by either α-GalCer or α-C-GalCer, further improved clinical arthritis symptoms, suggesting that the therapeutic effect of both glycolipid antigens was hampered by IFN-γ in the early phase of the disease. Although no phenotypic changes in conventional T cells or antigen presenting cells could be detected, T cell cytokine production after in vivo administration of anti-CD3 14 days after onset of clinical arthritis was profoundly distinct between α-GalCer versus the C-glycoside analogue treated animals. Whereas α-GalCer treated mice produced substantially higher amounts of IL-10 upon systemic anti-CD3 stimulation compared to PBS controls, T cells from α-C-GalCer treated mice, by contrast, produced substantially lower levels of cytokines, suggesting a general T cell hyporesponsiveness. In conclusion, these findings suggest long-term, ligand-specific and partially IFN-γ dependent immuno-modulatory effects of iNKT cells in a mouse model of joint inflammation.

017 PROBABLE RHEUMATOID ARTHRITIS METHOTREXATE VERSUS PLACEBO THERAPY (PROMPT)-STUDY: INDICATIONS FOR A WINDOW OF OPPORTUNITY IN THE TREATMENT OF PATIENTS WITH ANTIBODIES AGAINST CYCLIC CITRULLINATED PEPTIDES

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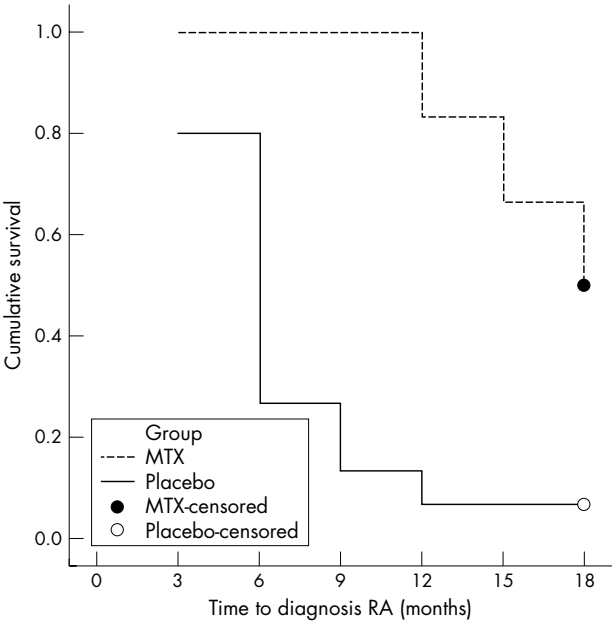
Aim: To determine whether patients with probable rheumatoid arthritis (ProRA) with or without the presence of antibodies against cyclic citrullinated peptides (anti-CCP) benefit from treatment with methotrexate (MTX). The main outcomes were diagnosis RA based on ACR 1987 classification criteria and progression of radiographic joint damage.
Methods: The PROMPT-study was a prospective double-blind placebo-controlled randomised multicentre trial in 110 patients with undifferentiated arthritis (UA) who fulfilled the ACR 1958 criteria for ProRA. Treatment started with MTX 15 mg/wk, or the same number of placebo tablets and was dictated by 3-monthly calculations of the disease activity score (DAS), with the goal to achieve and maintain a DAS $\leqslant$2,4. After 12 months, the study medication was tapered to nil. As soon as a patient fulfilled the ACR 1987 RA classification criteria, the treatment was continued with verum MTX. Joint damage was scored on 6-monthly radiographs of hands and feet according to the Sharp/van der Heijde



Abstract 017 Kaplan-Meier survival analysis for the diagnosis RA. Left: Anti-CCP-positive group, p=0.0001. Right: Anti-CCP-negative group, p=0.51.

method by two independent observers, with the radiographs in chronological order and masked for patient identity. At the end of the study, the anti-CCP status was determined.
Results: In the MTX-group, less anti-CCP-positive patients developed RA compared to the placebo-group (fig). In the anti-CCP-negative patients no such benefit from early MTX-treatment was seen. Anti-CCP-negative patients showed less damage progression regardless of MTX or placebo treatment than anti-CCP-positive. Anti-CCP-positive patients treated with MTX had less damage progression than those treated with placebo.
Conclusion: Anti-CCP-positive patients with UA fulfilling the ACR 1958 criteria for probable RA seem to benefit most from treatment with MTX. Fewer patients develop RA according to the ACR 1987 criteria and they have less progression in radiographic joint damage. This indicates the existence of a window of opportunity in anti-CCP-positive arthritic patients to influence the disease progression into fullblown RA.

018 CD40/CD40L INTERACTIONS BETWEEN BONE MARROW HAEMOPOIETIC PROGENITOR AND STROMAL CELLS REPRESENT A CONTRIBUTORY MECHANISM TO THE INCREASED PROGENITOR CELL APOPTOSIS IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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Background: The CD40-CD40L molecules are over-expressed in peripheral blood mononuclear cells of patients with systemic lupus erythematosus (SLE) and soluble CD40L (sCD40L) levels are markedly increased in patients with active disease. The distribution of the CD40/CD40L dyad, however, on bone marrow (BM) stem/progenitor cells and BM stromal cells and the possible consequences of their interactions in patients' haemopoiesis is entirely unknown.
Objective: To evaluate the expression of CD40/CD40L in the BM $CD34^+$ cells and marrow microenvironment cells, respectively, in patients with SLE and investigate the possible involvement of CD40/CD40L interactions in the apoptotic depletion of haemopoietic stem/progenitor cells previously reported in SLE.
Patients and Methods: BM samples from from posterior iliac crest aspirates were taken after informed consent from 17 patients with SLE and 20 age- and sex-matched healthy volunteers. The expression of CD40 in the $CD34^+$ cell fraction of the BM mononuclear cells (BMMCs) was evaluated by 2-colour flow-cytometry. The role of CD40 in the survival characteristics of BM $CD34^+$ cells was studied (a) by evaluating the proportion of apoptotic cells in the $CD34^+/CD40^+$ and $CD34^+/CD40^-$ cell fraction by means of flow-cytometry and 7-aminoactinomycin D (7-AAD), (b) by enumerating the clonogenic cells in the BMMC fraction following 14-day incubation with recombinant human (rh) CD40L by means of clonogenic assays in methylcellulose. The expression of CD40L in the BM microenvironment cells was evaluated by studying CD40L mRNA expression in long-term BM culture (LTBMC) adherent layers and immunomagnetically sorted $CD3^+$ and $CD14^+$ BMMCs by RT-PCR and cytokine production in LTBMC supernatants by ELISA.
Results: CD40 was minimally expressed on normal $CD34^+$ cells but was highly expressed in the $CD34^+$ cell fraction of patients with SLE ($P<0.001$). Among the $CD34^+$ cells, the $CD40^+$ cell fraction contained statistically significant higher proportion of apoptotic cells, in comparison to the $CD40^-$ cell fraction, in both SLE patients and healthy controls ($P<0.001$ and $P<0.001$, respectively) suggesting that the CD40 molecule is probably implicated in the apoptotic process of BM $CD34^+$ cells. In keeping with this finding was the decrease in the clonogenic progenitor cell number of patient BMMCs following incubation with rhCD40L compared to baseline ($P<0.002$).

No statistically significant difference was found between patients and controls in the CD40L mRNA expression in the adherent layer of LTBMCs or the isolated $CD3^+$ and $CD14^+$ BMMCs. However, in protein level, patient LTBMC supernatants contained statistically significant increased sCD40L compared to healthy controls ($P<0.05$). In specific, 50% of SLE patients and none of the controls displayed detectable CD40L levels in LTBMC supernatants.
Conclusion: Patients with SLE display increased CD40 expression in their BM $CD34^+$ cells and enhanced CD40L production by BM microenvironment cells. The CD40/CD40L interaction represents a novel mechanism contributing to the accelerated apoptosis of BM stem/progenitor cells in these patients.

019 MICROCHIMERISM AND HLA GENES ANALYSIS IN A FRENCH SCLERODERMA COHORT

J. Rak[1], P. Pagni[1], Y. Allanore[2], K. Tiev[3], J. Cabane[3], J. R. Harlé[4], D. Launay[5], E. Hachulla[5], D. Farge-Bancel[6], S. Parlier[6], A. Kahan[2], D. Reviron[7], J. Roudier[1,8], N. C. Lambert[1]. *[1]INSERM U639, Marseille; [2]Service de rhumatologie, Hôpital Cochin, Paris; [3]Service de Médecine Interne, Hôpital St Antoine, Paris; [4]Service de Médecine Interne, Hôpital La Conception, Marseille; [5]Hôpital Claude Huriez, Lille; [6]Service de Médecine Interne et pathologie vasculaire, Hôpital St Louis, Paris; [7]Etablissement Français du Sang; [8]Service de Rhumatologie, Hôpital La Conception, Marseille*

Background: Systemic sclerosis or scleroderma (SSc) is a rare autoimmune disease characterised by excessive collagen deposition in the skin and internal organs and microvascular injury. The predilection of SSc for women in their childbearing post years and the clinical similarities with chronic graft-versus host disease, a known condition of chimerism (donor cells in host), led to the hypothesis that a certain type of chimerism might trigger to SSc. Interestingly, studies in prenatal diagnosis revealed the existence of cells arising from pregnancy persisting for decades after delivery creating fetal microchimerism (FMc) in the mother and maternal Mc in her progeny. More frequent and quantitatively greater FMc has been detected in women with SSc compared to healthy women confirming an interesting new field of research for this disease. Although persistence of Mc in healthy individuals suggests that Mc per se is not a risk factor, but could be pathogenic in the context of other risk factors such as genetic susceptibility, HLA relationship among host and non-host cells.
Objectives: We propose to test on a French SSc cohort the hypothesis that FMC has a role on the pathogenesis of SSc in a particular HLA context.
Methods: In collaboration with five French hospitals, we recruited 138 patients with SSc, including 26 men and 112 women. Clinical data include history of pregnancies, date of diagnosis, type of SSc (limited or diffuse) and type of autoantibodies. DNA extracted from whole blood samples and/or peripheral blood mononuclear cells was HLA typed for HLA-DRB1, DQA1, DQB1 alleles. We tested 48 SSc women and 10 healthy women for male Mc by a Y chromosome specific Real Time PCR (DYS14).
Results: Among the 120 subjects typed for HLA-DRB1 the analysis is restricted to women because of the small number of men. Our preliminary results on HLA–DR associations confirm a significant correlation between HLA-DRB1*11 alleles and patients with a diffuse disease (30,9%) compared to those with a limited disease (6,8%). The HLA-DRB1*1104 allele is particularly increased in patients with anti-topoisomerase autoantibodies (40,5%) compared to patients with anti-centromere autoantibodies (0%).

Among women who gave birth to at least one male before the onset of the disease, male DNA was present in 41% of patients and a 25% of controls.
Conclusion: These preliminary results are still under investigations but highlight the presence of more frequent Mc in SSc women and the importance of clinical data in the overall analysis. Further studies, such as correlating HLA genotyping, trans generational HLA relationship and quantities of Mc, are needed to understand the functional role of Mc in SSc.

020 MICROCHIMERISM AND HLA GENES ANALYSIS IN A FRENCH SCLERODERMA COHORT

J. Rak[1], P. Pagni[1], Y. Allanore[2], K. Tiev[3], J. Cabane[3], J. R. Harlé[4], D. Launay[5], E. Hachulla[5], D. Farge-Bancel[6], S. Parlier[6], A. Kahan[2], D. Reviron[7], J. Roudier[1,8], N. C. Lambert[1]. *[1]INSERM U639, Marseille; [2]Service de rhumatologie, Hôpital Cochin, Paris; [3]Service de Médecine Interne, Hôpital St Antoine, Paris; [4]Service de Médecine Interne, Hôpital La Conception, Marseille; [5]Hôpital Claude Huriez, Lille; [6]Service de Médecine Interne et pathologie vasculaire, Hôpital St Louis, Paris; [7]Etablissement Français du Sang; [8]Service de Rhumatologie, Hôpital La Conception, Marseille*

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quantitatively greater FMc has been detected in women with SSc compared to healthy women confirming an interesting new field of research for this disease. Although persistence of Mc in healthy individuals suggests that Mc per se is not a risk factor, but could be pathogenic in the context of other risk factors such as genetic susceptibility, HLA relationship among host and non-host cells.
Objectives: We propose to test on a French SSc cohort the hypothesis that FMC has a role on the pathogenesis of SSc in a particular HLA context.
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021 INCREASED EXPRESSION OF CHEMOKINE-LIKE RECEPTOR 1 AND CHEMERIN IN RHEUMATOID ARTHRITIS PATIENTS

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Background: Rheumatoid arthritis (RA) synovium is characterised by a dense infiltrate, consisting of macrophages, T and B cells, plasma cells, neutrophils and dendritic cells (DC). Inflammatory chemokines present in RA synovium may contribute to the accumulation of these immune cells.

Chemerin is a potent chemotactic agent that was recently identified as the ligand of the G-protein-linked receptor chemokine-like receptor 1 (CMKLR1). This receptor is specifically expressed by tissue macrophages, circulating plasmacytoid (p)DC and immature monocyte-derived DC. Chemerin is expressed by many tissues, including spleen, lymph nodes, tonsils and inflamed skin. However, the expression of chemerin and its receptor, CMKLR1, has not yet been investigated in the inflamed joint. Since we have recently observed that the RA synovium is enriched with pDC, we investigated whether the chemerin/CMKLR1 system might play a role in attracting pDC to the inflamed joint.
Methods: Synovial tissue was obtained from 14 patients with RA, 8 with psoriatic arthritis (PsA) and 8 with inflammatory osteoarthritis (OA). Immunohistochemistry of synovial tissue was performed using specific antibodies against chemerin and CMKLR1 and stained sections were evaluated by digital image analysis. Chemerin activity (chemotaxis) in synovial fluids (SF) was achieved by investigating the ability of CMKLR1-transfectants to migrate towards SF. To identify the C-terminal processing of chemerin, chemerin from RA SF was purified using heparin chromotography, followed by PAGE separation, MALDI-TOF and mass spectra (tandem MS/MS analysis).
Results: Chemerin and CMKLR1 expression in RA ST was significantly higher compared with PsA+OA ST. Chemerin expression was confined to blood vessels whereas CMKLR1 expression was observed in dispersed cells throughout the synovial sublining. In all diagnostic groups studied, SF activity and production of chemerin was observed. Identification of the C-terminal of chemerin in RA demonstrated that its sequence is the same as the form identified in serum.
Conclusion: The in vivo distribution of chemerin in RA ST, located at the luminal side of inflamed blood vessels, strongly suggests that chemerin is involved in the migration and accumulation of pDC and macrophages into the inflamed joint. Since pDC do not respond to inflammatory chemokines, chemerin is likely the key chemoattractant responsible for the migration of pDC into the synovium. Blocking the novel chemerin/CMKLR1 system represents an attractive candidate for future drug development by disrupting disease perpetuation.

022 CD28NULL T CELLS ARE INCREASED IN PERIPHERAL BLOOD AND PRESENT IN THE INFLAMED MUSCLE OF PATIENTS WITH POLYMYOSITIS AND DERMATOMYOSITIS

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Background: The inflamed muscle tissue of patients with idiopathic inflammatory myopathies (IIMs) is infiltrated by T cells. In dermatomyositis (DM) CD4+ T cells are dominating, in polymyositis (PM) CD8+ T cells. In a number of other autoimmune diseases CD4+ CD28null T cells are found with increased frequency in the peripheral blood (PB) compared to healthy controls. In chronic virus infections CD8+CD28null T cells are increased. CD28null T cells are oligoclonally expanded T cells lacking the co-stimulatory molecule CD28, but have acquired new stimulatory molecules. We have previously shown that CD4+CD28null T cells from patients with rheumatoid arthritis (RA) are rapidly and more easily activated to proliferate and to produce TNF and IFN-gamma than conventional CD28+ T cells. Further, in some inflammatory disorders high frequencies of CD4+ CD28null T cells have been associated with a more severe disease.
Aim: To investigate the frequency of CD28null T cells in peripheral blood and inflamed muscle tissue from patients with IIM, and study the in vitro effector functions of these cells.
Materials and Methods: Peripheral blood from 23 patients with DM, 39 with PM and 40 healthy controls (HC) was screened for CD4+ and CD8+ CD28null T cells by FACS. The presence of CD28null T cells in muscle tissue was elucidated by three-colour immunofluorescence microscopy. Proliferation and secreted cytokines were measured on sorted T cells subsets after anti-CD3 in vitro stimulation.
Results: Both disease groups had significantly higher frequencies of CD4+ CD28null T cells in PB, median DM 8%, PM 4.9%, HC, 1%, $p<0.0001$ and $p=0.002$ respectively. CD8+ CD28null T cells were significantly increased in PB of patients with PM, median 52% compared to HC, 29.5%, $p<0.05$. Both CD4+ and CD8+ CD28null T cells could be detected in the infiltrates of inflamed muscle tissue from patients. Functional assays show that CD4+CD28null T cells from patients with IIM are less prone to proliferate compared to the CD28+ counterparts. However CD28null T cells, both CD4 and CD8, show an immediate in vitro hyperresponsiveness by secreting TNF and IFN-γ at stimulation levels that did not trigger conventional CD28+ T cells.
Conclusions: The fact that repeated antigen stimulation induces loss of CD28 expression by T cells and increased frequency of CD28null T cells in a number of autoimmune diseases, makes it tempting to speculate that repeated encounter of autoantigens generate the CD28null T cells. Our data show that CD28null T cells, potent secretors of IFN-γ and TNF, are present in the inflamed muscle tissue and enriched in peripheral blood of patients with PM and DM. This indicates a role of CD28null T cells in the pathogenesis of idiopathic inflammatory myopathies.

023 THE INDUCTION OF T CELLS WITH REGULATORY PHENOTYPE IN EXPERIMENTAL ARTHRITIS BY IL-18

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Aim: Interleukin-18 (IL-18) apparently plays a dual role in arthritis. IL-18 is known to stimulate Th1 maturation and to promote collagen-induced arthritis (CIA) when given during immunisation. However, we observed in previous studies that blocking IL-18 in DBA/1J mice with established CIA results in exacerbation of the disease. The present study is undertaken to investigate whether this anti-inflammatory role of IL-18 in mice suffering from collagen-induced arthritis is mediated by the induction of T cells with regulatory characteristics.
Methods: DBA/1J mice were immunised at day 0 and 21 with bovine collagen type II resulting in a gradual onset of CIA. Murine CD3+ spleen T cells were isolated by magnetic-activated cell sorting (MACS) separation. Subsequently, CD3+ T cells were stimulated for 3 days with 100 ng/ml IL-18 or with a combination of IL-18, anti-CD3 and

anti-CD28 monoclonal antibodies. Determination of regulatory T cell markers, like CD4+CD25bright expression and the up-regulation of the forkhead/winged helix transcription factor Foxp3 were determined by flow cytometry and at the mRNA level. To evaluate the effect of mIL-18 on ongoing arthritis, DBA/1J mice with established disease on day 22 were systemically injected with 3×10e8 ffu adenovirus encoding IL-18.
Results: IL-18 induced a CD4+CD25bright T cell population and up-regulated Foxp3 expression after stimulation of spleen CD3+ T cells derived from mice with active collagen arthritis. In contrast, CD3+ T cells from naive DBA/1J mice stimulated with IL-18 showed no up-regulation of regulatory T cell markers. However, stimulation of these T cells with a combination of IL-18, anti-CD3 and anti-CD28 did lead to the induction of a CD4+CD25bright T cell population. These results suggest that a co-stimulation is necessary for the induction of a regulatory T cell phenotype by IL-18 in T cells from naive mice. Moreover, it argues that T cells from arthritic mice were already primed in vivo to shift to T cells with a regulatory phenotype after IL-18 stimulation. Interestingly, systemic adenoviral overexpression of IL-18 in mice with established disease led to the development of a less severe collagen-induced arthritis as compared to control virus treated mice. In addition, CD3+ spleen T cells from such IL-18 treated immunized mice showed a shift from T-bet (Thelper 1 marker) to Foxp3 mRNA expression, indicating that IL-18 promotes the induction of T cells with regulatory characteristics.
Conclusion: With this study, we demonstrate that IL-18 is able to induce a regulatory phenotype in spleen derived CD3+ T cells which is characterised by Foxp3 and CD25bright induction. These results suggest that IL-18 might play a dual role in arthritis by having pro- and anti-inflammatory properties via T cells.

024 PROTECTION AGAINST COLLAGEN INDUCED ARTHRITIS WITH DIFFERENTIALLY MODULATED DENDRITIC CELLS

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Introduction: Dendritic cells (DC) are crucial for the initiation of T cell immunity and therefore play an important role in the initiation and regulation of immune responses in arthritis. Full mobilisation of effector T cells depends on the proper maturation of DC. Current evidence indicates that the type of T cell response induced is crucially dependent on the activation status of the DC. In this study, we explored the immunologic effects of differentially matured DC on the development of collagen induced arthritis (CIA), a well defined model for RA.
Methods: CIA is induced after injection of bovine collagen type II (CII) in CFA. It is a typical B cell-mediated autoimmune disease, as CII-specific antibodies are sufficient and required for disease induction. Especially IgG2a antibodies are thought to be important, as IgG2a is able to efficiently recruit effector-mechanisms.

Here, we investigated the possibility to protect mice against arthritis in an Ag-specific manner employing differentially modulated dendritic cells (DC).
Results: DC modulated with TNF, IL-10 or dexamethasone, but not LPS activated DC, were able to decrease disease severity and incidence. Even in a therapeutic setting, when measurable autoantibodies (against murine CII) are present in serum, IL-10 modulated DC are capable of suppressing CIA. Moreover, protection against disease correlates with lower anti-mCII IgG2a/IgG1 ratios compared to control mice. However, differentially modulated DC installed different modes of protection, since T cell responses, induced by these differentially modulated DC were different. TNF and IL-10 stimulated DC skews T cells towards the Th2 cell subset, whereas dexamethasone modulated DC induce the production of IL-10 production without the concomitant production of IL-5.
Conclusion: Thus, these data indicate that targeting DC represent an effective way to modulate arthritis and indicate that different types of DC, although leading to similar clinical effects, mediate protection via different mechanisms.

025 T CELLS SHOW IMMUNOREGULATORY FUNCTION FOLLOWING CONTACT WITH BIP TREATED DENDRITIC CELLS

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Background: High expression of the endoplasmic reticulum chaperone BiP is found in the rheumatoid synovium and cell-free BiP is present in the synovial fluid and blood of RA patients. In vitro and in vivo studies in the murine collagen induced arthritis model have shown that extracellular BiP has immunoregulatory functions that counteract inflammation. Since BiP binds to peripheral blood monocytes (MO) we investigated the effect BiP had on MO differentiation into dendritic cells (DC) and the subsequent changes in T cell function following interaction with BiP treated DC.
Methods: MO were negatively selected using immunomagnetic beads and cultured with interleukin-4 and granulocyte macrophage-colony stimulating factor for 7 days to induce differentiation into immature dendritic cells (iDC) in the presence and absence of BiP. Development of mature dendritic cells (mDC) was induced by the addition of lipopolysaccharide for the final two days. Purified allogeneic or autologous T cells were placed in co-culture with iDC or mDC for 4 days and then washed and re-cultured with irradiated autologous peripheral blood mononuclear cells and fresh autologous T cells, with or without anti-CD3 antibody, for a further 3 days. Phenotypic changes were analysed by immunofluorescence and flow cytometry while functional changes, such as cytokine production and lymphocyte proliferation, were quantified by ELISA or uptake of tritiated thymidine, respectively.
Results: Differentiation of MO into iDC in the presence of BiP caused few significant changes in phenotype other than a tendency to increased expression of CD14+ and CD40+CD85j+ and to decreased expression of IL-10R+. BiP inhibited maturation of DC, as determined by expression of CD83 (mDC, 38.6±10.6 versus mDC(BiP) 10±4.7), and maintained high CD14 and low IL-10R expression. IDC(BiP) and mDC(BiP) produced significantly more IL-10 than iDC ($p=0.013$) and mDC ($p=0.008$) respectively. Preliminary data showed that allogeneic T cells pre-cultured with BiP-treated iDC or mDC, when placed in autologous culture, inhibited fresh T cell responses to anti-CD3 antibody by 66.5±5% and 38.7±20.1%, respectively, while autologous T cells pre-cultured with iDC inhibit fresh T cell responses by 65.4±23%.
Conclusion: Maturation of MO into DC in the presence of BiP is altered to enhance T cell development with regulatory function.

026 ELEVATED SOLUBLE ST2 AND CYTOKINE LEVELS IN SYNOVIAL FLUIDS OF PATIENTS WITH INFLAMMATORY SYNOVITIS

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ST2 is a member of the IL1 receptor family widely reported to have a purely negative regulatory function on IL1R and TLR signalling. However, recent reports have identified a novel cytokine (IL-33) that binds ST2L (the membrane bound isoform), activates NFkB and induces Th2-type cytokine responses. The soluble cleaved isoform of ST2 (sST2) acts as an ST2L receptor antagonist by competing for the IL-33 ligand, and may therefore have an antagonistic effect on induction of Th2 responses. We quantified the level of sST2 present in synovial fluid (SF) from patients with inflammatory synovial disease (rheumatoid, psoriatic and septic arthritis and ankylosing spondylitis (RA, PsA, SA and AS) respectively) using a sensitive capture ELISA. Data were compared with SF from non-inflammatory osteo-arthritis patients (OA). Quantitative ELISA demonstrated that SF from RA, PsA, SA and AS all showed significantly elevated levels of sST2 ($p<0.05$) in comparison with OA patients. SF cytokine expression in a cohort of each group was quantified using Luminex. Levels of TNFa and IL-12 were significantly elevated in RA and PsA in comparison with OA SF ($p<0.05$). No significant correlation was observed between sST2 levels and TNFa expression, though there was evidence that increased sST2 correlates with increased IFNa. These data indicate that sST2 is associated with joint inflammation. As inflammatory synovitis is a result of a polarised Th1-type T cell response, sST2 may contribute to disease through inhibition of IL-33-driven Th2 responses.

027 EXPRESSION AND ACTIVITY OF INDOLEAMINE 2,3 DIOXYGENASE IN ARTHRITIS

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The enzyme indoleamine 2,3 dioxygenase (IDO) is thought to contribute to the generation of immune tolerance. The tolerogenic action of IDO is mediated by two mechanisms: depletion of its substrate, the essential aminoacid tryptophan, and generation of a number of tryptophan catabolites that affect the activity of T cells. Therefore, dysregulation of IDO activity can provide a potential mechanism for the tolerance

breakdown that characterises autoimmune diseases, such as rheumatoid arthritis.

Our objective was to analyse the effects of blocking IDO function on the progression of collagen-induced arthritis (CIA) in DBA/1 mice as a well-established animal model of rheumatoid arthritis.

Arthritis was induced in male DBA/1 mice by intradermal injection with bovine type II collagen (CII) in CFA. IDO activity was suppressed in vivo by subcutaneous injections of 1-methyl-tryptophan (1-MT; a specific inhibitor of IDO), starting at the time of arthritis onset. Ten days after arthritis onset, mice were sacrificed and serum, spleen and spinal cord were collected to determine IDO expression levels by RT-PCR. In addition, IDO activity was determined by photometric detection of its substrate, kynurenine. Anti-collagen antibody levels in serum were determined by ELISA and T cell responses were measured by culturing lymph node cells in the presence of CII or anti-CD3 antibody. Proliferation and cytokine production were evaluated by tritiated-thymidine incorporation and capture ELISA, respectively. For assessment of IDO expression and function in vitro, dendritic cells were stimulated with IFNg and CTLA-4-Ig and T cells were stimulated with IFNg and anti-CD3/CD28 antibodies. IDO expression was then determined by RT-PCR and its function was assessed by fluorometric determination of tryptophan concentrations in culture supernatants.

Treatment of splenic cultures in vitro with 1-MT increased proliferative responses to anti-CD3 in arthritic mice, but not in non-immunised mice, suggesting that IDO was expressed in CIA. This was supported by the analysis of IDO expression in lymphoid organs which showed the presence of IDO mRNA during the course of CIA. Treatment of arthritic mice in vivo with 1-MT resulted in inhibition of IDO activity, as determined by reduced kynurenine levels in serum. Clinical score and paw swelling was increased in 1-MT treated animals compared to vehicle-treated animals, indicating that IDO inhibition promotes a more severe arthritis. Specific anti-CII antibody levels and the anti-collagen IgG2a/IgG1 ratio were not affected by treatment with 1-MT. The proliferation of cultured lymph node cells from 1-MT treated mice was increased in response to collagen but not to anti-CD3 antibodies, suggesting an antigen-specific effect of IDO in the inhibition of T cell responses.

In summary, our data provide evidence that IDO is acting as an immunoregulatory molecule in CIA and future work will address the question of whether over-expression of IDO leads to down-regulation of disease.

028 THE MOLECULAR BASIS OF FUNCTIONAL STABILITY OF REGULATORY T CELLS: THE MEMORY OF TH CELLS FOR IL-10 EXPRESSION IS CONDITIONAL UNLESS THE GENE IS IMPRINTED BY GATA-3

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Regulatory T lymphocytes appear to be potent tools for the targeted therapy of inflammatory rheumatic diseases. Their regeneration in vivo or their adoptive transfer into rheumatic patients are currently discussed as therapeutic options. Success will critically depend on the stability of their regulatory phenotype at the site of interaction with the proinflammatory immune reaction. Here we analyse the memory of Th memory lymphocytes for the reexpression of the cytokine interleukin-10 (IL-10), which is a critical function of regulatory T lymphocytes. The memory of Th lymphocytes for expression of the cytokines IL-4 and IFN-γ is dependent on costimulation of the activated cells by IL-4 and IL-12, respectively. It is established already after primary stimulation of naïve Th lymphocytes, i.e. in later reactivations of the Th cells, expression of IL-4 or IFNγ occurs independent of the original costimuli. Here we show that the expression of IL-10 is induced by either IL-4 or IFNγ/IL-12 costimulation, and remains dependent on these costimulations in reactivated Th cells, i.e. the memory for IL-10 is not stable and reexpression of IL-10 remains conditional. When isolated ex vivo by the cytometric cytokine secretion assay, IL-10 expressing Th lymphocytes lack a stable memory for IL-10. Such cells would be useless for therapeutic applications, unless instructive conditions for IL-10 expression could be achieved at the presumptive site of action simultaneously. Alternatively, a stable memory for IL-10 expression under any, even adverse conditions, can be established in Th lymphocytes by repeated stimulation with antigen and the instructive signal IL-4. We show here, that this is due to IL-4 induced, elevated expression levels of the transcription factor GATA-3. We have identified a regulatory element in the IL-10 promoter which is addressed by GATA-3 in IL-10 expressing Th cells. GATA-3 induces epigenetic remodelling of the IL-10 gene, which might be prerequisite for a stable IL-10 memory.

029 EXPANSION OF INHIBITORY RECEPTOR EXPRESSING NKR+ T CELLS IN PATIENTS WITH RHEUMATOID ARTHRITIS IN CLINICAL REMISSION FOLLOWING ANTI-TNFα THERAPY

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Aim: The disease modulating effects of anti-tumour necrosis factor-alpha (anti-TNFα) therapies have been well described in rheumatoid arthritis (RA). However the effect of anti-TNFα therapies on the immune system has not been systematically studied. Natural killer receptor expressing (NKR+) T cells are increasingly recognised as playing an important role in the pathogenesis of autoimmunity and were the focus of this study.
Methods: NKR+ T cell populations were studied in the peripheral blood of age and sex-matched subjects with (a) RA in clinical remission (DAS28$<$2.6) following anti-TNFα therapy (n = 15), (b) active RA on traditional DMARDS (n = 15) and (c) healthy controls (HC) (n = 15). Differences between the groups were assessed using the Mann-Whitney U test where $p<0.05$ was deemed significant.
Results: Small but significant subpopulations of T cells expressing NK receptors, including CD94 and NKG2A, were seen in healthy controls (mean 2.56% and 2.6% of total CD3+ cells respectively) and patients with active RA (mean 2.16% and 2.19%). Expansion of this population was seen in patients in clinical remission following anti-TNFα therapies (mean 4.13% and 4.14%) ($p<0.05$). A strong correlation was seen between expression of these receptors in remission ($r = 0.629$, $p<0.05$) and HC ($r = 0.687$, $p<0.05$). Poor correlation was seen in patients with active RA ($r = 0.436$, $p = 0.104$). Anti-TNFα therapy also expanded CD8+ cells (mean 16.66% of lymphocytes), almost normalising (mean 20.06% in HC) this depleted population in active RA (mean 10.25%) ($p<0.05$)
Conclusions: These data suggest that anti-TNFα therapy modulates NKR+ and CD8+ T cells. Expansion of CD94/NKG2A+ T cells following anti-TNFα therapies may suggest a novel mechanism of action for these therapies allowing down regulation of T cell receptor mediated functions including cytotoxicity and autoreactivity.

030 ACTIVATION OF THE INTERFERON-α PATHWAY IN PATIENTS WITH SJÖGREN'S SYNDROME: AN EXPLANATION FOR THE FAILURE OF ANTI-TNF AGENTS?

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Background: Recent evidence from clinical trials suggests anti-TNF agents are not effective in controlling Sjögren's syndrome (SS). In patients with juvenile rheumatoid arthritis, anti-TNF treatment has been shown to induce an over expression of interferon-alpha (IFN-α) inducible genes. IFN-α has recently been implicated in the pathogenesis of SS. Given the lack of efficacy of anti-TNF agents in SS we hypothesised that activation of the IFN pathway may underlie the inefficacy of such agents in SS.
Objective: To investigate the IFN activity in the plasma of patients with SS compared to healthy individuals, and examine IFN activity before and after treatment with etanercept.
Patients and Methods: Study population included 20 patients with SS according to the American-European Consensus Group criteria assigned to treatment with etanercept (25 mg twice a week) or placebo in the setting of a 12 week randomised double blind, placebo controlled clinical trial. IFN plasma activity was determined in the 20 patients with SS and 30 healthy controls using the following assay. Cells of the WISH epithelial cell line, which are highly responsive to type-I IFNs, are cultured with 50% patient plasma. Recombinant IFN-α and media are used as positive and negative controls respectively. mRNA is purified from the WISH cells, and reverse transcriptase is used to generate cDNAs of all mRNA transcripts. Quantitative real-time PCR is performed on the cDNA using primers for genes that are highly induced by IFN-α. The housekeeping gene GAPDH is also analysed in the samples to control for background gene expression, and melt curve analysis and standard curves are used to ensure specificity and accuracy. The IFN-α induced genes are compared with housekeeping gene expression to determine relative expression. This method has proven to be highly sensitive and reliable for the detection of IFN-α in plasma.
Results: A significant increase in plasma IFN activity was noted in SS patients at baseline compared to healthy controls ($p<0.0001$). Furthermore, 7 out of 10 etanercept treated patients had an additional increase in IFN-a induced gene expression after treatment compared to 2 out of 10 of placebo treated patients ($p<0.025$).

Conclusions: Type I IFN activity is detectable in the plasma of many patients with SS compared to healthy controls. Anti-TNF treatment seems to exacerbate the IFN pathway activation in these patients, providing a possible explanation for the lack of efficacy of these agents in SS.

031 THE ANALYSIS OF SYNOVIAL EXPRESSON PROFILES FROM LASER CAPTURE MICRO-DISSECTED CELLS

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Aims: Rheumatoid arthritis is a complex disease, most likely with multiple causes and which shows also various pathological pathways. The inflammatory process is thought to be driven by macrophages and fibroblasts, though B cells and their secreted antibodies may have an important impact on the chronic inflammation. To understand the immune processes taking place in the inflamed tissue, methods were established to analyse gene expression in defined synovial tissue cell subsets.
Methods: Frozen tissue sections were prepared and stained with fluorescence labeled antibodies. Defined cell subsets were isolated by Laser Capture Microdissection (Arcturus), RNA was purified, cDNA transcribed and amplified. Gene expression profiles were determined by Affymetrix array hybridisation and data confirmed by Real Time PCR. Expression data were compared with profiles obtained for highly purified cell populations and analysed for functional profile components (FPCA).
Results: Using these methods we are able to obtain enough aRNA for gene expression analysis from less than 10 000 cells. Initial results have been obtained by isolating plasma cells accumulating in the inflamed synovial tissue and analysing their gene expression.
Conclusions: These methods open a new way to study directly ex vivo the immune processes taking place in the chronically inflamed synovial tissue of patients with rheumatoid arthritis. and this will be essential to gain an understanding of the chronic inflammation. A comparison of gene expression in various synovial tissue cells will help to tease apart the contribution of fibroblasts, macrophages, T and B cells to the complex pathogenesis of rheumatoid arthritis.

032 DIFFERENTIAL REQUIREMENTS OF NAÏVE PERIPHERAL BLOOD B CELLS FOR ACTIVATION BY CPG DNA

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Aim: The maintenance of B cell tolerance is critical to avoid autoimmune diseases such as systemic lupus erythematosus (SLE). B cell tolerance is an active process that needs to be capable of withstanding powerful co-stimulatory conditions such as the activation of Toll-like innate immune receptor 9 (TLR-9) by CpG DNA during the course of infections. One approach to assessing autoimmunity is to induce B cell activation and differentiation in vitro and enumerate auto-reactive B cells directed against specific antigens. We have modified a previously reported culture system using CpG DNA with sequential steps that allows for T cell independent activation of naïve $CD19^+CD27^-$ human peripheral blood B cells as well as $CD19^+CD27^{+\ memory}$ with plasmablast differentiation. In addition, this is the first in vitro system to document significant upregulation of CD138, a plasma cell marker.
Methods: Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation and $CD19^+$ B cells enriched and separated into $CD27^+$ and $CD27^-$ populations by magnetic bead affinity columns. Cells were then cultured in a three step system: (1) *Day 0–4*: CpG + IL-2/10/15; (2) *Day 4–7*: IL-2/6/10/15 + anti-CD40L; (3) *Day 7–10*: IL-6/15 + IFN-α + hepatocyte growth factor (HGF) + hyaluronic acid (HA). Cell phenotyping was performed at each stage by FACS analysis, intracytoplasmic immunoglobulin staining, and immunoglobulin secretion assay. FACS analysis was used to measure intracytoplasmic TLR-9 after stimulation with CpG DNA plus differing combinations of the cytokines, IL-2, IL-10 and IL-15. Genomic DNA was isolated from $CD138^+$ cells and amplified by PCR for V gene sequence analysis.
Results: The three step culture system induced activation and differentiation of both memory ($CD27^+$) and naïve ($CD27^-$) human B cells. Both naïve and memory populations displayed a plasma cell phenotype: $CD19^{low}$ $CD20^{low}CD27^+$ $CD38^+$ $HLA\text{-}DR^{low}$. With the use of IFN-α, HGF, and HA, all factors known to induce CD138 expression, up-regulation of CD138 was observed. 85% of the $CD27^-$ derived $CD138^+$ cells demonstrated a germline configuration without somatic mutations, confirming their origin from naïve precursors. The plasma cells derived from $CD27^+$ B cells are primarily IgG producing cells, whereas those plasma cells derived from naïve $CD27^-$ B cells are IgM producing plasma cells. Intracytoplasmic TLR-9 expression was present at baseline in both naïve and memory B cells. The amount of intracytoplasmic TLR-9 following CpG DNA activation was dependent on the protocol of cytokines used. The use of cytokines IL-2, IL-10 and IL-15 along with CpG DNA produced the most proliferation and the most expression of TLR-9 in both the naïve and memory cells. The physiologic response of naïve B cells to CpG DNA remains to be determined.
Conclusion: TLR-9 stimulation via CpG DNA can induce in vitro activation and plasmablast differentiation in naïve $CD27^-$ B cells and memory $CD27^+$ B cells. Using our culture system we hope to gain insights into the linkage between innate and adaptive immunity and its relevance to B cell tolerance. Such knowledge will be instrumental in improving our understanding of autoimmunity in general, and specifically SLE.

Autoimmunity

033 EPSTEIN-BARR VIRUS EBNA-1 AND GP110 PROTEINS ARE THE TARGET OF HIGH ANTIBODY RESPONSES IN RHEUMATOID ARTHRITIS PATIENTS EXPRESSING THE SHARED EPITOPE

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Objectives: More than 90% of adults worldwide have been infected with the Epstein-Barr virus (EBV) and carry the virus as a life-long persistent infection. In patients with RA (rheumatoid arthritis), T cell responses to EBV gp110, a protein of the replicative cycle, are decreased. In addition, EBV load is increased 10 fold in RA PBMCs. Finally, RA patients' sera contain high titre antibodies to EBV antigens. We undertook identification of the targets of chronic antibody responses.
Patients and Methods: 146 patients and 82 healthy controls were enrolled. We studied antibody responses to purified Epstein-Barr nuclear antigen 1 (EBNA-1) and gp110 (latent and replicative proteins respectively) by ELISA.
Results: Overall, no difference was found between RA patients and controls. However shared epitope positive (SE+) RA patients had higher antibody titres to EBNA-1 and gp110 than shared epitope negatif (SE-) patients and SE+ controls. Conversely, SE- RA patients had lower antibody titres than SE- controls.
Conclusion: High antibody responses to EBV Viral Capsid Antigen (VCA) and EBNA complexes antigens have been described 20 years ago by immunofluorescence. Here, by ELISA on purified proteins EBNA-1 and gp110, we show that SE+ RA have higher antibody responses than SE- RA patients.
Acknowledgement: ARP and SFR.

034 MINOR SALIVARY GLAND EPITHELIAL CELLS: A SOURCE OF THE IMMUNOREGULATORY HORMONE ADIPONECTIN

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Introduction: Primary Sjogren's syndrome (pSS) is an autoimmune disease characterised by lymphocytic infiltrates associated with destruction and dysfunction of salivary and lacrymal glands. The functional epithelia of these glands have been shown to be aberrant activated. In pSS patients, adipocytes that are present in certain minor salivary gland (MSG) biopsies, are found in association with fibrosis a fact which connotes a healing process. Adiponectin is a hormone that is predominantly produced by adipose tissue and displays immunoregulatory properties. Therefore, we examined the expression of adiponectin in MSG biopsies of pSS patients and controls.
Patients and Methods: Immunohistochemistry was applied for the study of adiponectin expression in paraffin-embedded MSG biopsy samples obtained from 15 pSS patients and 15 control subjects. In addition, Reverse Transcriptase-Polymerase chain reaction was used to amplify the adiponectin gene product, as well as the two adiponectin receptors AdipoR1 and AdipoR2 mRNAs in cultured non-neoplastic salivary gland epithelial cells (SGEC) lines derived from 4 patients with primary SS, and 4 control subjects. In these cultured cell lines immunoblotting was also used to detect the expression of adiponectin protein.
Results: Immunohistochemistry for adiponectin revealed positive staining in the adipocytes of the lesions. Additionally, positive expression of adiponectin was also noted among ductal epithelial cells in pSS and

controls, as well as in infiltrating lymphocytes in pSS. RT-PCR products analysis yielded a 230-bp fragment of adiponectin gene in the pSS, as well in the control SGEC samples. The specific band was sequenced to confirm that it represents the actual product. The expression of AdipoR1 and AdipoR2 receptors genes were also detected in all mRNA samples from pSS patients and controls. By immunoblotting, adiponectin protein expression was detected in cultured SGEC only from pSS patients (4/4), but not from controls.
Conclusion: Our results provide novel evidence that adiponectin is produced by ductal epithelial cells of MSG, and has probably autocrine and paracrine effects, as indicated by the concurrent expression of the relevant receptors. The high constitutive expression of adiponectin protein by the SGEC of pSS patients likely owes to the aberrant activation of these cells. Finally, our findings likely indicate that adiponectin may act as an anti-inflammatory agent counterbalancing the local inflammatory reactions, or as an anti-apoptotic factor preventing programmed cell death in ductal epithelial cells.

035 SERUM AMYLOID A IN AUTOIMMUNE THROMBOSIS

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Background: Serum amyloid A (SAA) has previously been reported to inhibit thrombin-induced platelet aggregation, indicating a protective role in vascular injury and the development of thrombi. It is currently unknown whether levels of serum SAA in arterial and/or venous thrombosis are elevated in comparison to other inflammatory markers C-reactive protein (CRP) and interleukin-6 (IL-6) and if these levels differ in the presence of autoimmune disease, specifically antiphospholipid syndrome (APS).
Objectives: The main objectives of this study were to (1) determine how levels of SAA, high-sensitivity CRP and IL-6 correlate to autoimmune diseases in patients with and/or without thrombosis, and (2) to discuss the parameters/relationships that influence the relative SAA values.
Methods: SAA, high-sensitivity CRP and IL-6 concentrations were determined by enzyme linked immunosorbent assay (ELISA). 84 patients with secondary APS (SAPS), primary APS (PAPS), systemic lupus erythematosus (SLE), SLE with antiphospholipid antibodies (aPL), venous thrombosis (VT) and arterial thrombosis (AT) were compared to healthy donors (n = 60).
Results: The percentages of patients above cut-off regarding SAA values were highest in the SAPS (9/17), SLE (8/16) and SLE + aPL (10/16) groups. Significant differences were observed between healthy donors and inflammatory groups of patients (SAPS and SLE +aPL) in all three measured parameters, while their values in non-inflammatory groups (PAPS, AT and VT) showed non-significant differences as compared to healthy donors. Using the Spearman correlation analysis we showed SAA and CRP to be correlated to a greater extent in SAPS patients (Rho = 0.76) than in SLE+aPL patients (Rho = 0.61), while SAA and IL-6 levels were analysed and found not to be correlated significantly in any of the groups. Ratios of SAA/CRP and SAA/IL-6 were determined and showed a wide distribution in the inflammatory groups (SAPS, SLE+aPL and SLE).
Conclusions: In summary, this cross-sectional, retrospective, small study and accompanying additional clinical considerations limit the ability to make definite conclusions. SAA would not serve as a useful systemic marker for venous, arterial thrombosis or PAPS (pro-coagulant events). It could however, be a good predictor of progression from a solely hypercoagulable thrombotic event to an overt highly inflammatory condition.

036 INHIBITION OF BPI'S ANTIBIOTIC FUNCTION BY BPI-ANCA IN DISEASES ASSOCIATED WITH CHRONIC INFECTION BY GRAM-NEGATIVE BACTERIA

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Background: The bactericidal/permeability increasing protein (BPI), a potent antimicrobial protein against Gram-negative bacteria (GNB) has been identified as target antigen of ANCA besides proteinase 3 or myeloperoxidase. BPI-ANCA are associated with Gram-negative chronic infection and can occur in reactive arthritis, inflammatory bowel disease (IBD), cystic fibrosis (CF) and TAP-deficiency, a syndrome resembling Wegener's Granulomatosis.
Aims: Our aim was 1. To investigate the ability of BPI-ANCA from patients with CF, IBD and TAP-Deficiency, to inhibit the antimicrobial activity of recombinant BPI (rBPI) in vitro. 2. To define BPI-ANCA linear epitopes and epitope spreading of BPI-ANCA in CF. 3. To analyse the association of BPI-ANCA levels with disease activity.
Methods: IgG from 24 IBD, 23 CF and 6 TAP-deficient patients were purified from sera (Protein G FPLC) and BPI-ANCA-levels measured in ELISA. Patients' IgG interaction with rBPI was assessed in a bacterial growth inhibition assay with E. coli DH5α. BPI-ANCA linear epitopes were determined using 246 synthetic 13mer peptides on cellulose membrane with an overlap of 2 aminoacids. Measurement of BPI-ANCA levels of 19 CF patients was performed on three occasions during a time interval of 6 years and inhibition of BPI and epitope spreading were followed up after 12 months. To compare experimental findings with clinical parameters (CRP, lung function, CDAI, bacterial colonisation) Chi-square analysis and Mann-Whitney-Tests were used.
Results: 37 of 53 BPI-ANCA preparations could inhibit BPI's antibiotic activity. Up to 12 epitopes per preparation were identified on the functionally important N-terminal as well as on the C-terminal half of BPI. No recurring epitope pattern was evident. In 8 of 19 CF patients epitope spreading occurred during the course of disease. BPI-ANCA levels were associated with disease activity (CDAI in Crohn's disease) and with a worsening lung function and bacterial colonisation in CF during the follow up period.
Conclusions: BPI-ANCA impair bacterial clearance in vitro and thus might contribute to maintained inflammation in vivo in diseases associated with chronic Gram-negative infection. Epitope analysis demonstrated epitope spreading of BPI-ANCA in CF suggesting that BPI-ANCA might contribute to pathogenesis with a prognostic significance.

037 ENHANCED CYTOKINE PRODUCTION BY DENDRITIC CELLS UPON TOLL-LIKE RECEPTOR STIMULATION AND ELEVATED SERUM LEVELS OF T CELL ATTRACTING CHEMOKINES IN SYSTEMIC SCLEROSIS

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Background: Systemic sclerosis (SSc) is an auto-immune disease in which T cells are thought to play an important role. Cytokines and chemokines produced by dendritic cells (DC) largely determine the type of T cell responses. In this study, we present novel data that suggest a role for DC and their inflammatory mediators in SSc.
Methods: We measured serum levels of the T cell attracting chemokines CCL17, CCL18 and CXCL16 in patients with SSc and healthy controls. Dendritic cells were cultured from monocytes of both SSc patients and controls. These cells were studied on their phenotype and production of TNF-α, IL-6, IL-10, IL-12, CXCL16, CCL17 and CCL18 upon TLR mediated stimulation.
Results: Circulating levels of CCL17, CCL18 and CXCL16 were significantly elevated in patients with SSc compared to healthy controls. No differences in DC phenotype were observed between DCs from patients and controls. Stimulation of DC with TLR4 ligands did result in a higher secretion of CCL17, CCL18 and CXCL16 in SSc whereas in contrast, the production of TNF-α, IL-6 and IL-10 by DC from SSc patients was significantly higher compared with that by DC from healthy controls.
Conclusion: Our data provide evidence for CXCL16 as a novel marker and confirm the importance of CCL17 and CCL18 in SSc. Furthermore, our data suggest a role for DC and TLR signaling in SSc pathology.

038 HIGH DOSE CHEMOTHERAPY EFFECTIVELY DEPLETES SHORT-LIVED, AUTOREACTIVE PLASMABLASTS IN PATIENTS WITH RHEUMATOID ARTHRITIS

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Aim: Circulating autoantibodies are a typical finding in patients with rheumatoid arthritis (RA). Production of these autoantibodies is derived from either short-lived, proliferating or terminally differentiated antibody producing cells. Proliferation is a key difference between these two subpopulations of antibody-producing cells. It is not known yet which cells are mainly responsible for autoantibody production in RA patients. High dose chemotherapy followed by autologous stem cell transplantation (HDC+HSCT) is an experimental therapy for RA patients and is specifically targeted to eliminate proliferating cells. The present study was conducted to investigate the contribution of proliferating versus non-proliferating (auto)antibody producing cells in RA.
Material and Methods: Eight RA patients treated with HDC+HSCT were immunized twice before and after treatment with a neoantigen (Rabies, RB) and a recall antigen (tetanus toxoid, TT). All patients were

followed for up to 2 years after treatment. The effects of HDC+HSCT on circulating B-cell and T-cell counts were measured by flowcytometry. Synovial biopsies were taken before treatment and 3 months after treatment to score infiltrating plasma cells. Serum titers of total immunoglobulins and autoantibodies (IgM-rheumatoid factor (IgM-RF) and IgG-cyclic citrullinated peptide (IgG-CCP)) were measured before treatment and during follow-up. Serum titers of circulating antigens (IgG tetanus toxoid (IgG-TT), IgM-rabies (IgM-RB) and IgG-rabies (IgG-RB)) were measured before and after each immunisation. Effects of HDC+HSCT on circulating B-cell and T-cell counts were measured by flowcytometry.

Results: HDC+HSCT resulted in complete depletion of CD19+ B-cells and CD20+ B-cells from the circulation in all (8/8) patients and significantly reduced circulating CD3+ T-cells. Histological analysis showed that the (mean ± SEM) number of plasma cells at baseline (35,6±19,3) were almost completely eradicated from the synovial tissue after HDC+HSCT (1,4±0,91 ; p=0.028). Titers of total IgM in the peripheral blood were more affected by HDC+HSCT than titers of total IgG . Serum titers of IgM-RF and IgG-CCP decreased after HDC+HSCT and were significantly correlated with decreases in total IgM and IgG respectively (β=0,74, p<0,0001; β=0,21, p=0,04 respectively). In one CCP-positive patient seroconversion of IgG-CCP was observed. After vaccination with rabies and tetanus, IgM-RB and IgG-RB rose and then decreased to baseline values after HDC+HSCT, while IgG-TT remained stable (p=0,013).

Conclusion: The present study shows that autoantibody production in patients with rheumatoid arthritis is largely dependent upon proliferating, short-lived antibody-producing cells.

039 UNDIFFERENTIATED CONNECTIVE TISSUE DISEASE WITH ANTI-RO/SSA ANTIBODIES: CLINICAL FEATURES AND ANTI-RO/SSA ISOTYPE DISTRIBUTION

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Background: Undifferentiated connective tissue disease (UCTD) are characterized by symptoms suggestive but not diagnostic for specific CTD and circulating autoantibodies against different nuclear antigens. Anti-Ro/SSA antibodies are detected in 12–30% of UCTD. Few data were reported about distribution or predictive value of different autoantibodies' isotype.

Aim: To determine the isotype and the titre variation of anti-Ro/SSA antibodies in a cohort of patients with UCTD.

Patients and Methods: 100 patients affected by UCTD, according to Mosca's criteria, with anti-Ro/SSA antibodies attending our outpatients clinic were studied. Antibodies to Ro/SSA were determined by CIE, using human spleen extract as substrate. The first and the last sera obtained from every subject at the diagnosis and during followup, respectively, were examined by ELISA (Pharmacia). All sera were tested using anti-human IgG, -IgM conjugated with horseradish peroxidase and -IgA conjugated with alkaline phosphatase.

Results: 100 patients, affected by UCTD, (7 males and 93 females), mean age of 51 years (SD 14.4), were followed for 7.6 years (SD 4.8). After a mean time of 6.9 years (SD 4), 35% of patients evolved in a specific CTD. Skin involvement, xerostomia and xerophtalmia were more frequently detected in evolved patients (p=0.0033; p=0.0013; p=0.035 respectively). Sera of evolved UCTD showed more frequently anti-dsDNA antibodies (p=0.048) and multiple antinuclear specificities (p=0.0044), while isolated anti-Ro/SSA (73% of cases) were prevalent in ''stable'' UCTD group (p=0.017). Most of sera showed reactivity to IgG anti-Ro/SSA antibodies (94%) as isolate isotype (40%) or in association with IgM or IgA, without difference between evolved and not evolved patients. Anti-IgA-Ro antibodies, globally detected in 39% and 47% at the onset and during follow up, were more frequent in ''stable'' UCTD, both in first and in second control (p=0.0039 and p=0.0083, respectively). The titre of anti-Ro antibodies did not significantly change between the first and the second sera, considering all Ig isotypes. By contrasts, comparing anti-Ro antibody titer of the first and the second sera in patients evolved in Sjögren's Syndrome and in stable UCTD, we found a significant increase of antibody concentration of IgG class only in patients who develop Sjögren's Syndrome (p=0.023).

Conclusions: Isolate anti-Ro/SSA antibodies, particularly IgA class, are prevalent in stable UCTD patients, without other ANA specificities. IgG anti-Ro/SSA titre tends to increase during follow up only in patients with UCTD developing Sjögren's syndrome.

040 HLA-DR GENES AND ANTI CALPASTATIN IMMNUNITY IN RHEUMATOID ARTHRITIS

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Most patients with rheumatoid arthritis (RA), a chronic inflammatory joint disease express particular HLA-DR alleles, like HLA-DRB1*0401, *0404, *0405, *0101, *0102, *1001. How these HLA-DRB1 alleles influence the development of RA is unknown.

To test whether HLA-DR alleles influence the production of specific autoantibodies in RA patients, we screened synovial proteins with sera of RA patients homozygous for HLA-DR alleles. Surprisingly, we observed that sera of RA patients homozygous for HLA-DRB1*0404 recognised a 100 kD synovial protein identified as calpastatin, the natural inhibitor of calpains (proteases involved in cartilage destruction). Autoantibodies against calpastatin have been previously described in rheumatoid arthritis but their specificity remains controversial.

To test the influence of different RA associated alleles on anti-calpastatin production, we calculated the frequency of positive sera in patients expressing either one RA associated HLA-DR allele or none by in-house ELISA using purified synovial calpastatin as immunosorbent. We observed that 23% of RA patients have antibodies to synovial calpastatin compared to 12% of controls. We found that RA associated HLA-DR alleles are associated with presence of autoantibodies to synovial calpastatin in RA patient's sera. Indeed, 29% of RA patients expressing RA associated alleles had autoantibodies to synovial calpastatin compared to 14% of RA patients with non RA associated alleles.

Then, we tested the interaction between calpastatin and HLA-DR alleles by a direct binding assay. Calpastatin comprises five domains of about 140 amino acids each called domain L, 1, 2, 3 and 4. We used 47 15 mer peptides encompassing the five domains of calpastatin for the binding to purified HLA-DRB1*0401, *0404, *0101 (RA associated alleles) and HLA-DRB1*0402, *0701 (non RA associated alleles). We found that every tested HLA-DR allele was capable to bind peptides from calpastatin. Peptides from domain 1 and 4 of calpastatin are the best HLA-DR alleles binders.

Finally, we tested T cell responses to calpastatin in RA patients. Preliminary results showed that peripheral blood T cells that recognise calpastatin are common in RA patients suggesting a strong immunity against calpastatin in RA patients.

041 IMMUNE RESPONSE TO 70 KD HEAT SHOCK PROTEINS AND RHEUMATOID ARTHRITIS

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Susceptibility to develop rheumatoid arthritis (RA) maps to a highly conserved amino acid motif expressed in the third hypervariable region of different HLA-DRB1 alleles. This motif is QKRAA, QRRAA, RRRAA. How this motif influences the development of RA is unknown. We previously observed that the QKRAA motif of HLA-DRB1*0401 carries an original function: it is a binding motif for 70 kD heat shock proteins. This is true for bacterial 70 kD hsp like DnaK and also at least one human hsp70, the constitutive 73 kD hsp, hsp73. These findings raise the question whether immunisation against bacterial hsp70s during bacterial infection can trigger immunisation against self hsp70 in people expressing HLA-DRB1*0401.

In this study, we tested if hsp70s can constitute RA triggering antigens after bacterial infection and if the abnormal reactivity is correlated with particular HLA-DR alleles. We found no evidence that HLA-DRB1*0401 can influence antibody and T cell responses to hsp70 in RA patients and controls. We then studied antibody and T cell responses to hsp70s in patients who developed arthritis after intravesical BCG therapy for bladder cancer. Intravesical therapy with BCG is a common procedure after bladder resection to prevent tumour proliferation. Patients with bladder carcinoma received repeated instillation of BCG to stimulate their immune system and destroy bladder tumour cells. In this context ''mimicking'' repeated bacterial infections, we observed that human hsp70s are the main target of cellular response after immunisation with BCG. This strong reactivity against human hsp70 is HLA-DR independent. Finally to check whether HLA-DR genes could influence the development of the immunity hsp70, we monitored antibody and T cell responses to hsp70s in patients after intravesical BCG therapy. We found that T cell precursor frequencies to human hsp70 is exaggerated in patients receiving BCG therapy but this strong reactivity is not associated by particular HLA-DR allele.

Thus, T cell responses against human hsp70s are common after BCG therapy and do not seem to be associated with arthritis even in

HLA-DRB1*0401 patients. Human hsp72 and hsp73 are T cell targets after BCG therapy, suggesting their potential use in vaccination.

042 HLA-DRB1*0404 IS MOST STRONGLY ASSOCIATED WITH ANTI CITRULLINATED FIBRINOGEN ANTIBODIES IN RHEUMATOID ARTHRITIS

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Rheumatoid arthritis (RA) develops in patients expressing particular HLA-DR alleles. RA patient's sera contain specific antibodies to a post translationally modified form of fibrinogen on which arginine residues are transformed into citrulline. We tested whether HLA-DR alleles influence the production of anti citrullinated fibrinogen antibodies (AFIBA) in RA patient's sera and whether the replacement of arginine by citrulline residues on fibrinogen peptides could modify their binding to RA associated HLA-DR molecules and their recognition by T cells in RA patients and controls.

To evaluate the influence of different RA associated alleles on AFIBA production, we calculated the frequency of AFIBA positive sera in patients expressing either one RA associated HLA-DR allele or none by in-house ELISA. We found that RA associated HLA-DR alleles are associated with presence of AFIBA in RA patient's sera. The RA associated HLA-DRB1*0404 allele is the most strongly associated with presence of AFIBA.

To test if citrullination could influence the HLA-DR-peptide interaction, we developed a direct binding assay. 53 15 mer peptides encompassing the whole alpha chain of fibrinogen and their 40 citrulline variants and 43 15 mer peptides encompassing the whole beta chain of fibrinogen and their 31 citrulline variants were tested for binding to purified HLA-DRB1*0401, *0404, *0101 (RA associated alleles) and HLA-DRB1*0402, *0701 (non RA associated alleles). Every tested allele was capable to bind peptides from the alpha and beta chains of fibrinogen. However, HLA-DRB1*0404 was by far the best fibrinogen binder. Citrullination didn't influence fibrinogen peptide binding to HLA-DR.

Finally, we tested T cell responses to native and citrullinated fibrinogen peptides in RA patients and controls. We found that peripheral blood T cells that recognise native or citrullinated fibrinogen peptides were common in RA patients and very uncommon in controls. Citrullination did not influence fibrinogen peptide recognition by T cells.

Thus, citrullination of fibrinogen has nothing to do with peptide/HLA-DR/T cell interaction. HLA-DRB1*0404 is highly associated with anti-citrullinated fibrinogen production because it binds the most fibrinogen peptides.

043 IDENTIFICATION OF SMALL HEAT-SHOCK PROTEIN B8 (HSP22) AS A NOVEL TOLL-LIKE RECEPTOR 4 LIGAND AND POTENTIAL INVOLVEMENT IN THE PATHOGENESIS OF RHEUMATOID ARTHRITIS

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Background: Dendritic cells (DC) are specialised antigen-presenting cells that can be activated upon pathogen recognition as well as recognition of endogenous ligands, which are released during inflammation and cell stress. Recognition of these ligands is facilitated by Toll-like receptors (TLR), which are abundantly expressed in synovial tissue from rheumatoid arthritis (RA) patients. Furthermore endogenous TLR ligands are found to be present in RA serum and synovial fluid and are significantly increased compared to serum and synovial fluid from healthy volunteers. The endogenous heat-shock protein 60 (HSP60) and HSP70, have already been subject of many studies. However, identification of novel endogenous TLR ligands might contribute to the elucidation of the role of TLR in RA.
Aim: To investigate whether 5 members of the small heat-shock protein (sHSP) family are involved in TLR4-mediated DC activation and whether these sHSP are present in RA synovial tissue.
Materials and Methods: Monocyte-derived DC from RA patients (N=7) and healthy volunteers (N=7) were cultured for 6 days and subsequently stimulated with recombinant αA-crystallin, αB-crystallin, HSP20, HSPB8 and HSP27. Although, LAL test results showed that all sHSP were endotoxin free, DC stimulation experiments were all performed in the presence of polymyxin B to exclude interference of bacterial endotoxins. To test the involvement of TLR4 in stimulation with these small heat-shock proteins, a TLR4 antagonist (E5564) was added to the culture. Phenotypical analysis of the DC was performed using flowcytometry and production of cytokines was measured using multiplex cytokine assays. Expression of HPSB8 and αA-crystallin in RA synovial tissue was determined by Western blot and immunohistochemistry.
Results: The small heat-shock proteins αA-crystallin and HSPB8 were able to induce DC activation and activate DC, as observed by a strongly increased production of pro-inflammatory cytokines and a mature phenotype. In contrast, αB-crystallin, HSP20 and HSP27 did not have these effects on DC. Intriguingly, using a TLR4 antagonist, we demonstrated that the activation of DC by αA-crystallin and HSPB8 was TLR4 dependent. Western blot and immunohistochemistry revealed that HSPB8 was abundantly expressed in synovial tissue from RA patients, whereas αA-crystallin could not be detected.
Conclusion: With these experiments we identified sHSP αA-crystallin and HSPB8 as two novel endogenous TLR4 ligands, from which HSPB8 is abundantly expressed in RA synovial tissue. These findings suggest a potential role for HSPB8 during the inflammatory process in RA.

044 CONCEQUENCES OF THE ASP299GLY TOLL-LIKE RECEPTOR 4 POLYMORPHISM IN RHEUMATOID ARTHRITIS

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Background: Toll-like receptors (TLR) are essential in the initial recognition of invading microorganisms. Nowadays, 11 members have been reported in mammals and among these TLR4 has been studied most intensively. Besides being involved in the recognition of microbial products from Gram-negative bacteria, such as lipopolysaccharide (LPS), TLR4 is also able to recognise endogenous molecules such as heat-shock proteins and fibronectin fragments. TLR4 is abundantly expressed in the synovial lining from rheumatoid arthritis (RA) patients and monocyte-derived dendritic cells from RA patients produce higher amounts of cytokines upon TLR4-mediated stimulation. The role of TLR in RA has recently been underscored by the finding that HSPB8, identified as an endogenous ligand for TLR4, is abundantly expressed in RA synovium (submitted). Furthermore, a genetic TLR4 variant, Asp299Gly, has been associated with decreased susceptibility for rheumatoid arthritis (RA).
Aim: To investigate whether the genetic TLR4 variant Asp299Gly has functional consequences, with respect to cytokine production.
Materials and Methods: PBMC from RA patients and healthy volunteers carrying or not carrying the genetic TLR4 variant, Asp299Gly (either heterozygous or homozygous), were stimulated with LPS (exogenous TLR4 ligand), HSPB8 (endogenous TLR4 ligand), pam3cys (synthetic TLR2 ligand) or medium alone. TLR2 and TLR4 mRNA expression before stimulation was determined using quantitative PCR. Cytokine production was measured using multiplex cytokine assays.
Results: TLR4 mRNA expression before stimulation was significantly decreased in cells from RA patients carrying the genetic TLR4 variant, whereas TLR4 mRNA expression in cells from healthy controls carrying this variant was not affected. TLR2 expression was equal for individuals carrying or not carrying the TLR4 variant. IL-6 and IL-10 production by the PBMC, upon TLR4 and TLR2 stimulation, were not different between cells from individuals carrying or not carrying the TLR4 variant. In addition, TLR4 mRNA expression was not correlated with the cytokine levels produced.
Conclusion: With these experiments we showed that, TLR4 mRNA expression is significantly reduced in PBMC from RA patients with the Asp299Gly TLR4 polymorphism. However, stimulation of the cells did not reveal in functional differences in terms of cytokine production. However, protein expression on the cell surface needs to be investigated to further clarify the relation between the genetic TLR4 variant, TLR expression and cytokine production.

045 BINDING OF HIGH AVIDITY ANTI-β2-GLYCOPROTEIN I ANTIBODIES DOES NOT DEPEND ON ANTIGEN DENSITY

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Introduction: Antibodies against β2-glycoprotein I (anti-β2-GPI) are generally believed to be of low avidity. The binding of anti-β2-GPI to physiologic concentrations of β2-GPI in fluid phase is weak due to their low avidity. In our recent study we demonstrated that anti-β2-GPI are also of high avidity. It has been proposed that high density

of immobilised antigen or its clustering is required for bivalent or multivalent antibody binding. We assume that the density of β2-GPI is important for low affinity antibodies because their avidity is increased by bivalent binding in vivo and in vitro. It is unlikely that the density of the antigen could have an important influence on the binding of high avidity antibodies or their high affinity Fab fragments and the detection by ELISA.
Aim: To evaluate the importance of β2-GPI concentration for binding high avidity anti-β2-GPI in ELISA.
Material:

- Plasma from a patient with antiphospholipid syndrome (APS), with high avidity anti-β2-GPI,
- IgG fraction from an APS patient, with high avidity anti-β2-GPI,
- affinity purified low avidity anti-β2-GPI of IgG class from an APS patient and
- Fab fragment prepared from affinity isolated, high avidity anti-β2-GPI from an APS patient.

Methods: Relative avidity was determined by chaotropic anti-β2-GPI ELISA with increased concentrations of NaCl. The Fab fragment was prepared by the controlled papain digestion.
Antigen density dependency was measured by anti-β2-GPI ELISA with increased concentrations of the antigen from 1.25 to 20 mg/l.
Results: High avidity anti-β2-GPI showed dose dependent binding to the antigen at the concentration range from 1.25 to 20 mg/l, regardless of the matrix (plasma, isolated IgG fraction in PBS). Similar results were obtained when using isolated Fab fragment of high avidity anti-β2-GPI. Isolated low avidity IgG anti-β2-GPI showed negligible binding to the antigen below 5 mg/l.
Conclusions: High antigen density resulting in bivalent binding is crucial for the detection of low avidity anti-β2-GPI, while high avidity anti-β2-GPI bind the antigen at low density monovalently.

046 PROTEINASE 3 INDUCES MATURATION OF DENDRITIC CELLS AND LICENCES THEM FOR A TH1 RESPONSE IN WEGENER'S GRANULOMATOSIS

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Aim: PR3 is the autoantigen in Wegener's Granulomatosis (WG), which is an autoimmune disease characterised by granuloma formation and vasculitis. However, it is not clear, how PR3 becomes target of adaptive immunity.
To address the question how proteinase 3 becomes target of adaptive immunity, we investigated the ability of PR3-maturated dendritic cells (DCs) to prime and expand autoantigen-specific CD4+ T cells in patients with WG and normal donors.
Methods: Purified CD4+ T cells from the same healthy controls (n = 5) or active WG patients (n = 5) were plated at 10*6 cells/well in flat-bottom 6-well plates in RPMI 1640 with 5% human serum. Enzymatically inactive PR3 as Ag (10 μg/ml) was added to DC culture medium on day 3. The following maturation-inducing agents were used in their respective optimised conditions on day 4, for 2 days: TNF-α (50 ng/ml), PR3 (10 μg/ml) or TNF-α + PR3 were added to the supplemented culture medium. Autologous DC cells prepared as above were added at 10:1 T/DC ratio. DCs and T-cells were cocultured for 24h. T-cells stimulated with staphylococcal enterotoxin B (SEB, 1 μg/ml) were used as a positive control. To detect the PR3 specific-T cell frequency we used a new technique based on the IFN-γ/IL-4 secretion capture assay (Miltenyi Biotec).
Results: We found that PR3 induces phenotypic and functional maturation of blood monocyte-derived immature DCs. Following either PR3- or TNF-α induced DC activation, cells differentiated only into INF-γ-producing cells, not IL-4 producing cells. Moreover, PR3-maturated DCs derived from WG patients induce a higher response of PR3-specific CD4+ T cells as compared to normal donors (HC: 0.16 +/−0.01% vs. WG: 0.72 +/− 0.11%, P = 0.02). Simultaneous stimulation with TNF-α and PR3 was not more potent than PR3 alone in inducing T cell activation. Thus, DCs become fully competent antigen presenting cells and can therefore induce stimulation of PR3-specific CD4+ T cells, which produce INF-γ and drive the polarisation towards the Th1-type phenotype typical of WG.
Conclusion: Wegener's autoantigen triggers DC maturation and licences DCs for a Th1 response that is critical to granuloma formation in WG. Our study provides first evidence that the autoantigen (PR3) plays an important role in the initiation of an adaptive immune response that finally may lead to the production of PR3-ANCA.

047 COMPARATIVE ANALYSIS OF T CELL ACTIVATION AND CLINICAL DISEASE ACTIVITY IN SYSTEMIC LUPUS ERYTHEMATODES PATIENTS

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Introduction: T cells from systemic lupus erythematodes (SLE) patients have a decreased proliferative capacity but at the same time display phenotypic signs of activation. Little, however, is known whether increased T cells activity results from an intrinsic alteration of T cell reactivity which might correlate and thus help to explain fluctuations in clinical disease activity or allow to detect activated auto-reactive T cells.
Methods: We therefore performed comparative analysis of the expression of surface marker molecules that are associated with early (CD69, CD25) or later (CD71, HLA-DR, CD45RO, CD62L, CD95) stages of T cell activation on peripheral blood CD4+ T cells from SLE patients by flow cytometrie (FACS) with the European Consensus Lupus Activity Measurement (ECLAM), the SLE disease activity index (SLEDAI) and SLE index score (SIS). In parallel we determined the proliferative capacity of CD4+ T cells from SLE patients as compared to HC.
Finally we performed time kinetic analysis of the expression of activation associated marker molecules on isolated CD4+ T cells upon stimulation in SLE patients as compared to HC.
Results: Phenotypic analysis revealed significantly increased proportions of CD4+CD69+, CD4+CD25+ and CD4+CD71+ T cells in SLE patients (n = 30) as compared to healthy controls (HC; n = 9). In addition, although not significantly, increased proportions of CD4+HLA-DR+, CD4+CD45RO+ and CD4+CD95+ but decreased proportions of CD4+CD62L+ were observed in SLE patients as compared to HC.
No significant correlation was observed for CD69 expression with clinical disease activity scores whereas a weak but significant correlation was observed for CD25, as well as for CD71 and HLA-DR expression with the ECLAM score whereas the SLEDAI score inversely correlated with the expression of CD62L. Although the proliferative capacitiy of CD4+ T cell was decreased in SLE patients, CD69, CD25, CD45RO and CD95 expression was upregulated and reached peak expression values earlier in SLE patients as compared to HC. In addition, a decrease in proportions of CD69+, CD25+ and CD62L+ was observed earlier in SLE patients as compared to HC. On the other hand, similar expression pattern were observed for CD71 or HLA-DR.
Conclusion: CD4+ T cells of SLE patients show an activated phenotype, and are characterised by an overexcitability but at the same time diminished proliferation and early phenotypic exhaustion upon stimulation. Comparative analysis suggest that clinical disease activity scores reflect chronic, rather than acute, stages of T cell activation. Analysis of T cell reactivity thus might help to monitor flares of disease activity and contribute to the understanding of mechanism involved in SLE pathogenesis.

048 SLE LYMPHOCYTES SHOW NORMAL EARLY RESPONSE TO GAMMA-CHAIN CYTOKINES, AS DETERMINED BY STAT 5 PHOSPHORYLATION

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Background: Peripheral lymphocytes of SLE patients are hyporesponsive to interleukin-2 (IL-2) and other γ-chain cytokines (IL-7, IL-15) with respect to proliferation and regulation of apoptosis. The intracellular signal transduction of these cytokines mainly involves the phosphorylation of Janus kinase 3 (Jak3) and Stat5 (Signal transducer and activator of transcription 5).
Methods: PBMC were of 20 SLE patients and 15 healthy controls were prepared. Phosphorylated Stat5 (pStat5) was measured in lymphocytes by fluorocytometry after intracellular staining with a directly labelled monoclonal antibody both ex vivo and after 30 minutes in medium with or without IL-2, IL-7, and IL-15. The surface expression of the three IL-2 receptor chains alpha (CD 25), beta (CD 122), and gamma (CD 132) was likewise determined by flow cytometry.
Results: The expression of the three components of the IL-2 receptor was similar between SLE patients and healthy individuals, and Stat5 phosphorylation was likewise not significantly different ex vivo (pStat5 mfi 36,89 ± 26,22 for SLE, 25,42 ± 19,24 for HC) or after 30 minutes in medium alone (25,34 ± 9,58 for SLE, 19,61 ± 8,24 for HC), and not dependent on disease activity and associated parameters. Importantly, and in contrast to effects on proliferation, lymphocytes of SLE patients had an intact early response comparable to the one of healthy individuals when stimulated with IL-2 (increase to 39,77 ± 17,7,

p<0.0001 for SLE, to 34,81±17,66, p<0.0005 for HC), IL-7 (to 40,65±18,44 for SLE, 37,88±17,12 for HC), and IL-15 (to 38,13±14,86, for SLE and 37,78±16,89 for HC).
Conclusions: In contrast to their proliferative and anti-apoptotic response, lymphocytes of SLE patients show a normal immediate response to γ-chain cytokines, as measured by Stat5 phosphorylation. The reduced proliferation might therefore be due to increased in vitro apoptosis of activated lymphocytes of SLE patients.

049 IN PRISTANE INDUCED ARTHRITIS HNRNP-A2 (RA33) IS A MAJOR AUTOANTIGEN OF POTENTIAL PATHOGENETIC RELEVANCE

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Background: In pristane-induced arthritis (PIA) severe erosive disease is induced in rats by a single injection of the non-immunogenic mineral oil pristane. Arthritis is assumed to be driven by autoreactive T cells but so far it has not been possible to link the immune response to joint antigens or other endogenous components. Apart from rheumatoid factors (RF), heat shock proteins (hsps), citrullinated proteins, glucose-6-phosphate isomerase (GPI) and hnRNP-A2 (i.e the RA33 antigen, A2/RA33) are being debated to be involved in the pathogenesis of RA and possibly also in PIA, but their precise role is still unresolved.
Aim: To analyse B- and T-cell responses to these RA associated autoantigens in rats with PIA.
Methods: IgG and IgM autoantibodies against IgG (RF), A2/RA33, GPI, citrullinated antigens, hsps and human autoantigens associated with other rheumatic diseases (such as SLE or scleroderma) were determined by immunoblotting, ELISA and line immuno assay in PIA-prone DA1.F rats. Cellular reactivity to RA associated antigens was determined by measuring in vitro IFN-γ, IL-4 and TNF-α secretion by T cells isolated from draining lymph nodes 10 days after pristane injection. Acute and chronic PIA was histologically examined by H&E, toluidine blue and tartrate-resistant acid phosphatase staining. Expression of A2/RA33 in joints and other organs was analysed by immunohistochemistry and Western blotting.
Results: Autoantibodies to A2/RA33 were detectable in rat sera already 5 days after pristane injection and reached maximum levels during acute phase. IgG-RF was elevated during acute phase, whereas IgM-RF was present already in naïve rats and did not change during progression of PIA. Apart from a few exceptions no autoantibodies to any of the other antigens were observed. T cells of all DA1.F rats tested secreted intermediate to high levels of IFN-γ and TNF-α upon stimulation with A2/RA33, whereas no IFN-γ was produced in response to other candidate antigens including citrullinated fibrin, hsps and GPI. Cytokine secreting T cells were $CD4^+$ thus showing a Th1 phenotype. Finally, pronounced overexpression of A2/RA33 in joints of rats suffering from acute and chronic PIA, but not in joints of healthy rats was revealed.
Conclusion: The presence of autoantibodies and autoreactive Th1 cells directed to A2/RA33 shortly after pristane injection in conjunction with synovial overexpression of A2/RA33 suggests involvement of this autoantigen in the pathogenesis of PIA. Thus, A2/RA33 seems to be one of the primary autoantigens in PIA and might therefore play a pathogenetic role in this model and possibly also in human RA.

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050 TISSUE TRANSGLUTAMINASE IN RHEUMATOID ARTHRITIS

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If food intake can modify the course of rheumatoid arthritis (RA) is an issue of continued scientific and public interest. Gluten free vegan diet has been shown to be of clinical benefit for certain RA patients[1] and that this benefit may be related to a reduction in immunoreactivity to food antigens. A minority of RA patients (5–7%) can suffer form other autoimmune diseases like coeliac disease (CD) and this supports the concept that changes in the gut immune system can influence or initiate an autoimmune disease. We have know investigated this topic in more detail.

We used an IgG, IgA anti-tissue transglutaminase antibody (anti-tTG) ELISA to assess the prevalence of anti-tTG in sera from 100 patients with RA, patients were tested for IgG and IgA autoantibodies to tTG. Moreover we tested anti-gliadin IgG and IgA, rheumatoid factor (RF) and anti-CCP in serum of patients with RA.

We found 53% RF, 56% with anti-CCP, 5% anit-tTG IgA, 6% anti-tTG IgG, 15% anti-gliadin IgA and 14% anti-gliadin IgG positive sera. In total 10% of all RA have anti-tTG antibodies and 22% have anti-gliadin antibodies. In total, 29% of all RA patients have either anti-tTG or anti-gliadin AK in the serum. Interestingly, all RA patients who were anti-tTG positive are also anti-CCP positive.

In addition we tested synovial fluid from 68 RA and 20 osteoarthritis patients (OA) and found that 38% of the RA had anit-tTG IgG/IgA, 15% had anti-IgA tTG but only 5% of the osteoarthritis group had anti-IgA tTG. Testing the serum of the same patients only 10% had anti-tTG IgG/IgA autoantibodies. Using tissue array we show that tTG is significantly overexpressed in in RA synovial tissue compaired to normal or OA tissue. When we modified protein extracts from synovial fibroblast cell clines with tTG we show that RA sera react with certain proteins of this extract, this clearly demonstrates that the tTG modified proteins are targeted by RA patient sera, and this may influence the immune complex formation in the joint.
Conclusion: It seems that tTG is not only an autoantigen involved in CD but its action in modifying different proteins may favour the concept that other autoantigens that are normally not part of an immune response can be modified and cause a self-aggressive immunologic response in RA, following the gliadin-initiated inflammatory response. This may explain that some patients with RA can benefit from a gliadin free diet.

1. **Hafstrom I**, Ringertz B, Spangberg A, *et al.* A vegan diet free of gluten improves the signs and symptoms of rheumatoid arthritis: the effects on arthritis correlate with a reduction in antibodies to food antigens. *Rheumatology (Oxford)* 2001;**40**:1175–9. PMID:11600749 [PubMed - indexed for MEDLINE].

051 CHANGES IN AVIDITY AND SPECIFICITY OF IGG DURING ELECTRO-OXIDATION. RELEVANCE OF BINDING OF ANTI-β2-GPI ANTIBODIES

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Aim: The immune response may be changed due to altered proteins or modifications of immunoglobulins, including oxidative processes. The susceptibility to oxidative modifications depends greatly on amino-acid moiety composition due to chemical characteristics (instability) of their side-chains. Initial steps of oxidation may change the specificity and avidity of immunoglobulins due to chemical alteration of the hypervariable region. We selected anti-β2 glycoprotein I autoantibodies (anti-β2-GPI) in order to assess their immunoreactivity to β2-glycoprotein I (β2-GPI). It is expected that anti-β2-GPI have a polar hypervariable region with high electron density due to cationic characteristics of the antigen. As such, they would be prone to redox changes.
Materials and Methods: 21 blood donors, tested to be negative for anti-β2-GPI. Another 20 selected anti-β2-GPI positive samples were chosen: 10 from the bank of more than 400 apparently healthy adult blood donors and 10 from patients with the antiphospholipid syndrome. The presence or absence of anti-β2-GPI was determined by in-house ELISA. Human sera were first affinity purified by protein G column and the isolated IgG fractions were exposed in phosphate buffer saline (pH=7,4) to direct current (DC)-mediated electromotive force using 9 and 4,5 V, (constant) for 15, 30, 60, 120, 180 and 300 seconds. The avidity of the antibodies was quantified by chaotropic ELISA with increased salt concentration in the binding buffer and determined as the relative immunoreactivity at 500 mM NaCl compared to 150 mM NaCl.
Results: Electro-oxidation of the IgG fraction from healthy persons leads to immunoreactivity to β2-glycoprotein I. Changes in the immunoreactivity as well as the avidity of antibodies against β2-GPI after being exposed to direct current were observed. Short term oxidations resulted in fluctuations of immunoreactivity in all 20 samples. After 300 sec of electro-oxidation at 4,5 V, 18 anti-β2-GPI positive samples showed a lower immunochemical reactivity in ELISA than the initial values and only 2/10 IgG fractions from APS patients were increased.
Conclusions: Oxidation is a normal process of all proteins including immunoglobulins. Prolonged oxidation leads to denaturation and loss of its biological activity which could have been the cause of the lower immunochemical reactivity in 18 anti-β2-GPI positive samples. Initial

steps of oxidation may change the specificity of immunoglobulins due to chemical alteration of the hypervariable region as shown by electro-oxidation of the IgG fraction from healthy persons. The oxidation of antibodies increases the hydrophilic nature of the paratopes and makes them more susceptible for the binding to cationic surfaces even without the strong surface-to-surface fitting. Inter-individual differences in the chemical stability of immunoglobulins and the patient's antioxidative status may influence a range of alterations and their impact on health/disease balance.

052 CLINICAL AND LABORATORY MANIFESTATIONS IN ANTI-MITOCHONDRIAL TYPE 5 ANTIBODY POSITIVE PATIENTS

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Background: From the first description of antimitochondrial antibodies type 5 (AMA M5), by Labro *et al* almost thirty years ago, their presence and significance were rarely reported. AMA M5 have been described in patients with high prevalence of thrombocytopenia, autoimmune haemolytic anaemia (AIHA), thromboses and recurrent fetal loss. It was shown that, although related to anticardiolipin (aCL), anti-β2 glycoprotein I (α-β2GPI) antibodies and lupus anticoagulant (LA), AMA M5 represents distinct serological marker of antiphospholipid syndrome (APS).
Objective: The aim of the study was to investigate the relevance of AMA M5 positivity, in particular, their relationship with the clinical and serological features of the disease.
Methods: Over the period of nine years in 79 (63 female, 16 male; mean age 41.9, range 9–75 years) patients' sera presence of AMA M5 were detected. Patients' sera were screened for typical M5 pattern by standard IIF technique on rat kidney sections. Concentration of aCL and α-β2GPI were determined by ELISA. Presence of LA in plasma was detected according to the international guidelines. VDRL was detected by standard test.
Results: The most common conditions were: in 22 SLE, 18 definite PAPS and 11 lupus-like disease. Haematological malignancies were diagnosed in 9 AMA M5 positive patients (5 non-Hodgkin lymphoma, 2 Waldenstrφm's macroglobulinaemia, 1myeloma multiplex and 1 chronic lymphatic leukaemia). Six patients had isolated AIHA and four had ITP. In the remaining 9 patients the following diagnoses were made: bicytopenia in two patients and TTP, RA, Sneddon's syndrome, epilepsy, systemic vasulitis, transverse myelitis, Sjögren's syndrome, each in one patient. Deep vein thromboses were observed in 14 patients followed by pulmonary embolism in five of them. Arterial thromboses were detected in 10 patients. The most prominent were neurological manifestations, observed in 24 patients: seizures in 10, strokes in 9, central venous thromboses in 3, TIA and transverse myelitis each in one patient, respectively. Recurrent fetal losses experienced 9 of 58 women. We observed a high prevalence of VDRL positivity (68%) and LA (58%) followed by thrombocytopenia (52%), IgM (67%) and IgG (55.7%) aCL. Anti-β2GPI IgG were detected in 27/64 and IgM in 25/64 patients. Positive Coombs' direct test was found in 26.6%. Presence of paraproteins was detected in 12 patients.
Conclusion: In this study we describe the clinical and laboratory features of the largest group of AMA M5 positive patients reported in literature to our knowledge. We have found that only one half of AMA M5 positive patients met the proposed criteria for the diagnosis of SLE or PAPS.

053 INADEQUATE ANTI-IDIOTYPIC RESPONSE, AS ASSESSED BY ANTIBODIES TO LA/SSB COMPLEMENTARY EPITOPES: A NOVEL COMPONENT IN THE DEVELOPMENT OF NEONATAL LUPUS

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Background: Neonatal lupus syndrome (NLS) occurs in a minority of children born to mothers positive for anti-Ro/La autoantibodies. Despite studies on subclass distribution of maternal and neonatal anti-Ro/La antibodies, or on the epitopes of Ro/La, the selectivity of the autoimmune response is still unknown. The anti-La/SSB response can be blocked by an active idiotypic (id)/anti-idiotypic (anti-id) network. Anti-id antibodies are identified using synthetic complementary epitopes to the major B-cell epitopes of La/SSB, generated from the complementary strand of La/SSB mRNA encoding for the B cell epitopes. Complementary epitopes bind anti-id antibodies in human sera, with high sensitivity and specificity.
Aim: The aim of this study was to evaluate the predictive role of idiotypic/anti-idiotypic response to major linear B-cell epitopes of La/SSB in pregnant women with anti-Ro/SSA and/or La/SSB antibodies in the development of NLS.
Materials and Methods: 63 sera of women, positive for anti-Ro/SSA and/or anti-La/SSB antibodies, collected during pregnancy or within 6 months after delivery, obtained from the Research Registry for Neonatal Lupus and PRIDE study (serial echocardiographs on anti-Ro positive pregnant women) and 30 serum samples from healthy blood donors were analysed blindly. Sera were tested by ELISA assays against synthetic peptides corresponding to major B-cell epitopes pep349-364 and pep289-308 of La/SSB, as well as against their complementary synthetic peptide analogues, cpep349-364 and cpep289-308 (anti-idiotypic activity). In order to identify hidden idiotypic antibodies, sera that did not exhibit idiotypic or anti-idiotypic activity against the major B cell epitope 349-364aa were subjected to heat treatment and incubation with the complementary epitope cpep349-364, followed by an ordinary anti-pep349-364 ELISA.
Results: After the completion of the assays, samples were decoded and individuals were categorised into three groups: Group A (n = 29): mothers carrying a child with NLS (cutaneous rash or heart block), Group B (n = 10): mothers with a previous child with NLS, but child exposed to current antibodies is healthy and Group C (n = 24): mothers giving birth to a healthy child and no history of a child with NLS. Mothers who had only healthy children exhibited higher anti-id activity (79.1%) compared to mothers pregnant with affected children (24.1%) ($P<0.0001$) or to mothers with history of NLS (30%) ($P=0.0151$). Moreover, 88.8% of the mothers of healthy children (Group B and C, n = 9) with no apparent anti-pep349-364 activity revealed in their sera hidden anti-pep349-364 response, blocked by anti-id antibodies as compared to 28.5% of women pregnant with an affected child (Group A, n = 14)($P=0.0094$).
Conclusion: The presence of anti-idiotypic antibodies to autoantibodies against the major B-cell epitope of La/SSB may protect the fetus from the harmful maternal autoantibodies, by blocking the $F(ab')_2$ fragment of anti-La/SSB antibodies. Thus the presence of anti-id antibodies may be a predictor of low-risk pregnancies for NLS development.

054 IN THE RAT, CITRULLINATION OF AUTOLOGOUS FIBRINOGEN MAKES IT IMMUNOGENIC, BUT THE INDUCED AUTOIMMUNE RESPONSE IS NOT SPONTANEOUSLY ARTHRITOGENIC

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Citrullination, the post translational conversion of arginyl into citrullyl residues by a peptidylarginine deiminase, is essential in the formation of the epitopes recognised by the rheumatoid arthritis (RA)-associated autoantibodies to citrullinated proteins (ACPA). Numerous arguments suggest that ACPA play an important role in RA pathophysiology. In particular, they are secreted by plasma cells of the RA synovial tissue where, as a result of the tissue inflammation, their major target, citrullinated fibrin, is abundant.

In the present study, we assessed the immunogenic and arthritogenic properties of autologous fibrinogen (rFBG) in female LEWIS (LEW), Brown-Norway and Dark-Agouti rats. The animals were inoculated with non-citrullinated (NC-rFBG) or citrullinated (C-rFBG) rFBG, emulsified in CFA. No antibody response was induced with NC-rFBG. In contrast, following a single injection of C-rFBG, an IgG autoimmune response developed that was mainly directed to the citrullinated form of rFBG but also directed to its native form. However, all the immunised rats, whatever their strain, remained devoid of any clinical and histological signs of arthritis up to 3 months after the first inoculation of C-rFBG.

Then, we tested whether the presence of an autoimmune response to C-rFBG could aggravate an arthritis triggered by a non-specific agent. In female LEW rats, intra-articular injection of incomplete Freund's adjuvant (IFA) induced an acute and transitory inflammation but this inflammation followed the same course in the absence and in the presence of anti-C-rFBG autoantibodies. However, formation of citrullinated fibrin in the IFA-injected joints could not be evidenced.

In conclusion, citrullination of rFBG allows breakdown of immunological tolerance but the autoimmune response developed is neither spontaneously arthritogenic nor able to aggravate a transitory acute arthritis. Whether the anti-C-rFBG response is able to aggravate an arthritis showing citrullinated fibrin deposits remains to be evaluated.

055 ALTERED FC GAMMA RECEPTOR EXPRESSION ON MONOCYTE SUBSETS IN RHEUMATOID ARTHRITIS

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Introduction: Interactions between IgG-containing immune complexes and Fc gamma receptors (FcγR) on peripheral blood monocytes may contribute to persistent synovial inflammation and joint destruction in rheumatoid arthritis (RA). FcγR are divided into 3 classes; FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). There are 3 isoforms of FcγRII; FcγRIIa (CD32a), FcγRIIb (CD32b) and FcγRIIc (CD32c). The balance of activatory (CD16, CD64, CD32a and CD32c) and inhibitory (CD32b) FcγR receptors on these cells might be of importance in the regulation of chronic immune complex-mediated responses in patients with RA. Human peripheral blood monocytes are a heterogeneous population which can be distinguished into classical CD14++ cells and a minor population of CD14low cells. Recent studies have noted changes in the proportion of the CD14low monocytes in patients with RA. However, previous studies have not comprehensively dissected out the monocyte subsets in relation to their expression levels of FcγR. In addition, confirming which activatory or inhibitory isoforms of CD32 are expressed on these monocyte subpopulations has been hampered due to a lack of specific antibodies.
Methods: The expression of CD16, CD64 and CD32a, b or c on CD14low and CD14++ subsets in normal controls (NC) and RA patients was determined by flow cytometry. Expression of CD32a was measured using antibody CIKM5. Expression of CD32b or CD32c could be demonstrated using antibody 3D3 in selected individuals. Binding of 3D3 to CD32a is abolished in individuals possessing a polymorphic variant of CD32a in which an arginine residue (CD32a-R131) is replaced with histidine at position 131 (CD32a-H131). Flow cytometry using 3D3 was therefore carried out in CD32a-H/H131 homozygous individuals to stain for CD32b and CD32c only.
Results: The expression of CD16 ($p=0.02$) and CD32 ($p=0.007$), but not CD64, was increased on CD14++ monocytes in RA compared to NC. The CD14low subset demonstrated consistently higher levels of CD16 ($p<0.001$) and lower levels of CD64 ($p<0.001$) compared to the CD14++ subset in both RA and NC. CD32a was expressed at high levels on both CD14low and CD14++ cells. In CD32a-H/H131 individuals, staining with 3D3 confirmed the presence of CD32b/CD32c on CD14low and CD14++ monocytes. Molecular analyses are underway to confirm the specific isoform of CD32 expressed in CD14low and CD14++ monocyte populations. In contrast to the previous literature, the CD14low subset was not expanded in RA.
Conclusions: Alterations in FcgammaR expression on the CD14++ monocyte population in RA may influence monocyte responsiveness to immune-complex mediated activation and contribute to aberrant inflammatory responses.

056 PAD4 IS UPREGULATED BY TNF-α IN ACTIVATED MACROPHAGES: A NEW MODEL FOR CYTOKINE DRIVEN AUTOIMMUNITY IN RHEUMATOID ARTHRITIS

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Background: Citrullination is increasingly being considered to be important in the pathology of rheumatoid arthritis. Proteins are citrullinated by peptidylarginine deiminases (PADs). Two isotypes are of particular interest: PAD2 and 4. These enzymes are expressed in immune cells including macrophages and granulocytes and are found in the joints of patients with inflammatory arthritis. Little is known about the regulation of these enzymes. In this study we set out to identify the effects of proinflammatory cytokines on the expression of PAD2 and 4 in the HL60 cell line. This cell line expresses both PAD2 and PAD4 and has a low level of endogenous citrullinated proteins, making it ideal for studies of regulation of PADs and citrullination of proteins.
Methods: HL60 cells were treated with TPA followed by IFN-γ to differentiate them to an activated macrophage phenotype. Differentiated or undifferentiated HL60 cells were treated with varying doses of TNF-α in the presence or absence of 100 U IFN-γ and incubated for 16 hrs. Cell lysates were analysed by immunoblot for the presence of PAD2 and 4 proteins. Total RNA was analysed for changes in the level of PAD2 and 4 mRNA by real time RT-PCR.
Results: TPA-differentiated HL60 cells treated with varying doses of TNF-α and 100 U/ml IFN-γ demonstrated a maximal 5.5 fold increase in levels of PAD4 mRNA at a dose of 5 ng/ml of TNF-α. This increase was mirrored by a parallel increase in the amount of PAD4 but not PAD2 protein detected by immunoblot. Treatment with TNF alone or IFN-γ alone had no effect on PAD2 or 4 protein and relatively minor effects (2.5 fold maximal increase) on mRNA. Undifferentiated HL60s did not show an increase in either PAD2 or 4 mRNA or protein levels when treated with TNF and IFN-γ.
Conclusions: This study demonstrates that treatment of cells with proinflammatory cytokines can modulate levels of PAD4. A combination of TNF and IFN-γ is required to achieve upregulation of PAD4 mRNA, resulting in expression of the protein. This increase is also dependent on the differentiation state of the cell, occurring only in those with an activated macrophage phenotype. The upregulation of PAD2 and 4 by proinflammatory cytokines may lead to the generation of novel citrullinated autoantigens during inflammation, causing a breach in tolerance and/or maintenance of disease in patients with RA. These data may also explain why TNF inhibition has such a profound disease modifying effect in RA, perhaps by inhibiting the generation of the autoantigens which drive it.

057 CHRONIC ARTHRITIS IN THE C57BL/6 MOUSE IS ASSOCIATED WITH A FAILURE TO CONTROL COLLAGEN SPECIFIC T CELL RESPONSES

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Rheumatoid arthritis is a chronic disease, characterised predominantly by synovial inflammation, and joint destruction. Pre-clinical assessment of therapeutics has relied largely on the collagen-induced arthritis (CIA) model in the DBA/1 (H-2q) mouse, in which mice are immunised with heterologous collagen type II (CII) in complete Freund's adjuvant (CFA). However, CIA in DBA/1 mice is a relatively acute disease characterised by a rapidly developing but short-lived immune response to collagen. It has recently been reported that C57BL/6 (H2b) mice also develop a form of arthritis after immunisation with chicken CII in CFA. This model is of particular interest because of the large number of transgenic strains that exist on this genetic background. We have found that CIA in C57BL/6 differs from DBA/1 mice in that it is a chronic disease. The aim of this study was to characterise CIA in the C57BL/6 strain, and to compare immune responses in C57BL/6 versus DBA/1 mice in order to identify mechanisms involved in driving chronic disease.

C57BL/6 were immunised with type II collagen, and disease incidence and severity was assessed and compared with CIA in the DBA/1 mouse. Lymph node responses and antibody levels to CII were assessed throughout the disease, in addition to evoked pain (allodynia). Histological characterisation of arthritic joints was performed, in order to characterise the nature of the inflammatory infiltrate the two strains of mice.

Immunisation of C57BL/6 mice with chicken CII resulted in arthritis that was less severe in clinical score and joint-swelling than in DBA/1 mice immunised with bovine CII. However, clinical disease and allodynia persisted in C57BL/6 mice for at least three months after onset but began to resolve in DBA/1 mice after approximately two weeks. Histological examination of the joints showed similar patterns of erosion, but inflammatory cells were not seen in the joints of DBA/1 mice after around 6 weeks post onset. In contrast, C57BL/6 joints showed pronounced mononuclear cell infiltrates throughout the period studied. Both C57BL/6 mice and DBA/1 mice developed T cell responses to chicken and bovine CII, respectively. However, only C57BL/6 mice developed a robust T cell response to mouse collagen, the response persisting throughout the study.

We have confirmed that a truly chronic form of arthritis can be induced in the C57BL/6 mouse, and that chronicity is associated with a robust and persistent T cell response to type II collagen. C57BL/6 mice will therefore be of great use in studying potential new therapeutics, especially those designed to inhibit the activity of effector T cells or promote the activity of regulatory T cell networks. Furthermore, the availability of transgenic strains on the C57BL/6 background offers enormous potential for probing mechanisms of disease pathogenesis which will further our understanding of human RA.

058 SPECIFIC REACTIVITY OF RHEUMATOID ARTHRITIS SERA WITH CITRULLINATED α-ENOLASE PEPTIDES STRENGTHENS THE EVIDENCE FOR THIS MODIFIED PROTEIN AS AN ANTIGEN IN RA

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Antibodies to citrullinated (deiminated) proteins are the most specific serological marker for rheumatoid arthritis (RA), present years before clinical onset and associated with a more destructive disease. These antibodies target citrullinated epitopes. However, it is not known which

protein(s) constitutes the antigenic source. Several candidate antigens have been proposed, including deiminated -fibrinogen, -vimentin and -collagen type II. In a previous study we identified citrullinated α-enolase as a novel candidate using immunoblotting. Here we have analysed the reactivity of a number of citrullinated α-enolase peptides with serum samples from RA patients, non-RA patients and healthy controls.

Based on our previously published mass spectrometry data, we initially designed and synthesised 9 citrullinated α-enolase peptides. These peptides cover 9 out of the 17 deiminatable arginine residues within α-enolase. Using ELISA, sera from 100 RA patients, 58 non-RA patients and 27 healthy controls were screened for reactivity with the nine peptides. Following identification of the immunodominant peptide, a corresponding control peptide was generated, which contained two arginines in place of the two citrulline residues. The anti-CCP status was also determined, using the commercially available anti-CCP2 kit (Eurodiagnostica, Malmo, Sweden).

59% of RA patients demonstrated an IgG immune response to one or more citrullinated α-enolase peptides. Interestingly, 8% of the rheumatoid patients with antibodies to citrullinated α-enolase were anti-CCP negative. A number of non-RA patients also reacted with the peptides. However, the level of reactivity was significantly lower and it is noteworthy that the two non-RA patients with the highest level of reactivity were also anti-CCP positive. No reactivity was observed in any of the healthy controls. Peptide 1, containing two citrulline residues, was identified as being immunodominant with a diagnostic sensitivity of 35% and a specificity of 95%. In a Swedish cohort of 40 RA patients, 30% were positive for peptide 1. In our study four RA-patients reacted with the arginine containing control sequence, while in the Swedish cohort none of the RA patients reacted with the control peptide.

Here we have built on our previous observations that RA patients have antibodies to citrullinated α-enolase. We have defined an immunodominant peptide, which is highly specific for the disease. Currently we are examining the possibility that the presence of antibodies to this epitope defines a clinical or immunogenetic subset of RA, in which citrullinated α-enolase has a pathogenic role. To investigate this hypothesis further, citrullinated α-enolase, as well as a KLH-conjugated peptide 1, will be used in experimental animal studies.

059 LOCALISATION AND CHARACTERISATION OF CITRULLINATED PROTEINS IN THE JOINTS FROM PATIENTS WITH ARTHRITIS

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Introduction: Over recent years it has become commonly accepted that antibodies to citrullinated proteins in the joint are mediators of the chronic inflammation observed in patients with rheumatoid arthritis (RA). However, it is still unclear which proteins harbour the epitopes targeted by these antibodies and where they are localised in the joint. We have recently characterised α-enolase as a candidate, though there are a number of other possible antigens including fibrinogen, vimentin and collagen. With others, we have previously reported the presence of citrullinated proteins in the rheumatoid synovial membrane. However, their presence in other compartments within the arthritic joint has not been studied. Here, we aimed to characterise further the citrullinated proteins localised in the synovial membrane, but also demonstrate the presence and character of those found in the synovial fluid, synovial fluid cells and cartilage.
Method: Citrullinated proteins were detected using an antibody specific for modified peptidylcitrulline. Expression in synovial fluid cells, separated by cyto-spinning, and synovial membrane sections was determined by immunocytochemistry and immunohistochemistry respectively. Whole cell lysis was performed on synovial membrane cells from patients with RA (n = 3), osteoarthritis (OA) (n = 3), juvenile idiopathic arthritis (n = 1) and psoriatic arthritis (n = 1). Proteins were extracted from cartilage of one patient with OA and one non-arthritic control. Synovial fluid from six patients with RA was treated with hyaluronidase. Samples were separated by one-dimensional SDS PAGE and the molecular weights of citrullinated polypeptides determined by immunoblotting.
Results: Citrullinated proteins were detected in all compartments of the joint. They localised to the nucleus of synovial fluid cells, to the cytoplasm of subsynovial cells and to the extracellular regions of the synovial membrane. In the synovial fluid a number of polypeptides reacted with the anti-citrulline antibody, most markedly at molecular weights of approximately 30, 50, 75 and 130 kDa. A range of citrullinated proteins were present in all synovial membrane cell lysates, but a triplet of bands between 50 and 75 kDa was most evident in RA samples. Strong reactivity of cartilage proteins with the anti-citrulline antibody was observed at 55, 65, 80 and 100 kDa.
Conclusions: Citrullinated proteins are not only found in the synovial membrane, but also in the cartilage, synovial fluid and synovial fluid cells. Here we have identified the molecular weights of a number of citrullinated proteins, expressed in arthritic joints, which we are currently characterising by 2-D gel electrophoresis and tandem mass spectrometry. Citrullinated proteins identified here, including nuclear proteins in synovial cells, previously undescribed, are potential autoantigens for driving autoimmunity in RA.
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060 FREQUENCY AND TYPE OF ANTINUCLEAR ANTIBODIES IN ADULTS AND CHILDREN WITH COELIAC DISEASE

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Background: Anti-endomysial (EMA) IgA antibodies are highly specific and sensitive serological marker for coeliac disease (CD). Coeliac disease, as an organ-specific autoimmune disease, can be associated with various organ-nonspecific autoantibodies. The aim of this study was to determine the frequency and type of antinuclear antibodies (ANA) in adults and children with CD.
Methods: From 2003 to 2005, 65 patients with newly diagnosed CD (33 adults and 32 children) were serologically analysed. The diagnoses were pathohistologically confirmed in 49/65 patients. The level of anti-EMA IgA and IgG antibodies were determined by indirect immunofluorescence (IIF) using monkey esophagus. The presence and titer of IgG ANA were detected by IIF using HEp-2 cells and rat liver as substrate. Anti–dsDNA IgG antibodies were detected by IIF on Crithidia lucilliae. The presence of anti-extractable nuclear antigen (ENA profile) antibodies was studied by commercial direct ELISA.
Results: All children (14 boys, 18 girls; mean age 7.1 years) were anti-EMA positive. 30/32 were EMA IgA positive (median titer 1:320). 2/32 had IgA deficiency and were anti-EMA IgG positive (median titer 1:320). All adult patients (4 males, 29 females; mean age 40.8 years) were anti-EMA IgA positive (median titer 1:320). 9/32 (28%) anti-EMA positive children were ANA positive (2 fine-speckled, 4 coarse-speckled, 3 few nuclear dots, median titer 1: 80); only 1/32 (3%) was positive for anti-ENA antibodies (Sm ++, RNP ++, SSA +++). 12/33 (37%) anti-EMA positive adults were ANA positive (5 homogenous, 7 speckled, median titer 1:80); 6/33 (18%) were positive for anti-ENA antibodies and 5/33 (15%) were anti-SSA +++. In children and adults, all anti-ENA positive patients were females. There were no anti-DNA positive patients in adults and children with CD. There were significant more females in adult CD ($p<0.01$). There were no differences in frequency and ANA titers ($p>0.05$). On the contrary, anti-ENA positivity was more frequently ($p<0$ .05) found in adult CD patients.
Conclusion: In comparison with children, ANA in adult patients with CD more frequently target determinants of ENA, especially SSA antigen. The mechanisms accounting for this epitope spreading in adult CD patients are not elucidated, but could include the duration of gluten exposition. In conclusion, ANA positive female patients with CD should be followed for manifestations of Sjögren's syndrome, systemic lupus erythematosus and subacute cutaneous lupus erythematosus.

061 THE EFFECT OF METHOTREXATE OR ANTI-TNF-α MONOTHERAPY ON IMPAIRED B CELL TOLERANCE IN RHEUMATOID ARTHRITIS

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Purpose: The role of pathogenic B cells in rheumatoid arthritis (RA) has reemerged in recent years, with a surging focus on B cell-depleting therapies such as rituximab. Yet the mainstays of treatment of RA remain methotrexate and the the anti-TNF agents – while little is known about their effect on the loss of B cell tolerance and the persistence of autoreactive B cells. Our recent work on treatment-naωve RA patients showed that neither of the bone marrow and the peripheral checkpoints, that remove autoreactive B cells in healthy donors, are functional in RA. We also identified 3 distinct subgroups of patients based on their repertoire patterns, possibly hinting at defects in immunoglobulin gene secondary recombination regulation in RA B cells. Our current aim is to determine if monotherapy with either methotrexate or anti-TNF-α agents have any effect on impaired early B cell counterselection that could help to explain the utility of these drugs in RA.

Methods: We obtained peripheral blood from patients who were clinically improved after months of monotherapy with either methotrexate (3 patients), or anti-TNF-α agents (1 patient each with etanercept and adalimumab), but without any adjuvant steroids in the months prior to phlebotomy. After isolating peripheral single B cells, we cloned and expressed in vitro antibodies from individual new emigrant (CD19+CD10+IgM+CD27-), and mature naive (CD19+CD10-IgM+CD27-) B cells. We examined by ELISA the reactivity of the recombinant autoantibodies by comparing them to our findings before treatment in those same patients, as well as to controls. In addition, we queried if the subgroup-defining antibody repertoires from the new emigrant fractions in treatment-naïve patients have resolved after months of therapy.
Results: The increased percentage of HEp-2-reactive and polyreactive B cells found in the pre-treatment patients is still present in the mature naοve B cell compartment after methotrexate or anti-TNF-α therapy. This further solidifies our previous conclusions from the pre-treatment patient analysis that revealed an early loss of B cell tolerance associated with RA. The decrease in secondary recombination which we found in the antibody repertoire patterns of some of these patients before treatment is still apparent in their post-treatment samples, also confirming our prior findings and suggesting that these agents have effents more downstream on B cell activation.
Conclusion: In RA, methotrexate and anti-TNF-α agents appear to act downstream from the impaired B cell tolerance checkpoints and do not correct the accumulation of autoreactive mature naïve B cells. Our new findings also strengthen our previous conclusions that there are distinct RA subgroups defined by their light chain repertoires, perhaps providing clues to how B cell receptor signaling is involved in RA pathogenesis.

062 CONCURRENT AUTOIMMUNE CONDITIONS IN A COHORT OF 205 PATIENTS WITH PRIMARY SJÖGREN'S SYNDROME

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Background: Primary Sjögren's syndrome (primary SS) is an autoimmune rheumatic disease characterised by lymphocytic infiltration of exocrine glands and epithelia in multiple sites. The involvement of lachrymal and salivary glands results in dysfunction leading to kerato-conjunctivitis sicca and stomatitis sicca. However, a significant proportion of patients with primary SS develop other manifestations involving internal organs, skin, blood and lymphatic systems, muscles and joints of the hands.
Methods: Two hundred and five patients attending the Sjögren Clinic at the University Hospital in Malmö, Sweden and fulfilling the 2002 EU/US consensus criteria were included. All clinical and laboratory data were recorded according to a standardised research protocol and stored in a database. Information was retrieved about thyroid disease, diabetes, celiac disease, and pernicious anaemia. For patients with thyroid disease and pernicious anaemia sera were examined for autoantibodies to thyroid peroxidase (TPO) and thyroglobulin (TG) (radio-immuno assay) and parietal cell antibodies (indirect immunofluorescence on sections of monkey stomach).
Results: Of the 205 patients with primary SS 44 (21%) had thyroid disease, 20 (10%) had pernicious anaemia, 3 (1.5%) had coeliac disease, and 1 (0.5%) had type 1 diabetes.

The determinations of specific autoantibodies showed that among the thyroid patients 54% had either anti-TPO or anti-TG. In the group with pernicious anaemia 50% had anti-parietal cell autoantibodies.
Conclusion: In this large cohort of patients with primary SS we found in accordance with previous observations that thyroid disease was particularly prevalent. However, pernicious anaemia was detected in a larger than expected proportion of patients. The figures for diabetes and coeliac disease were too small to draw any conclusions. Organ-specific autoantibodies were only detected in approximately 50% of the patients with clini-cally overt thyroid disease or vitamin B12 deficiency.

063 CITRULLINE SPECIFICITY IN NON-RA ANTI-CCP2 POSITIVE SERA

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Background: Autoantibodies directed against citrullinated proteins (e.g. anti-CCP) can be detected in rheumatoid arthritis (RA) patients with very high specificity. Combined literature data of the second generation anti-CCP (anti-CCP2) test show that 77% of RA patients are positive while the positivity percentage in non-RA disease controls is 5%. These antibodies can be detected years before the first clinical symptoms and have excellent diagnostic and good prognostic potential. Recently, in a large group of sera (n = 133) from Italian patients with type-1 autoimmune hepatitis (AIH-1), without clear rheumatoid arthritis overlap, a relatively high percentage (9%) of anti-CCP2 positivity was scored.
Aim: To investigate the nature of the observed anti-CCP2 positivity in AIH-1 sera and in other groups of non-RA disease patients (mainly psoriatic arthritis, palindromic rheumatism, systemic lupus erythematosus and Sjögren's syndrome).
Methods: Serum samples of mainly anti-CCP2 positive AIH-1 and other non-RA disease patients were recruited and tested for citrulline-specific reactivity using the Immunoscan RA second generation anti-CCP kit (Euro-Diagnostica) with the citrullinated as well as with the corresponding non-citrullinated (arginine-containing) antigen. For a selection of anti-CCP positive AIH-1 sera the reactivity was characterised in more detail by ELISA using a set of citrullinated peptides.
Results: About half of the anti-CCP2 positive AIH-1 sera reacted with the CCP2 epitope in a non-citrulline-specific fashion, whereas the citrulline-specificity in the non-RA rheumatologic disease group was 90%. Some of the anti-CCP2 positive AIH-1 sera also reacted with several other peptides (CCP1, vimentin, fibrinogen), but again in a non-citrulline specific manner. Control experiments ruled out that the reactivities observed were directed against the chemical structures used for immobilisation of the peptides or against BSA, another common factor in the procedure. Further analyses using truncated peptides and alanine substitution mutants demonstrated that the reactivities correlated with the amount of arginines and/or positive charge present in the antigen.
Conclusion: For some patients, in particular AIH-1 patients, non-citrulline dependent reactivity in the anti-CCP2 test can occur. Thus in such cases care should be taken in interpreting the results for diagnostic purposes. A control ELISA with a corresponding arginine-containing antigen is then needed to investigate the citrulline-specific recognition.

The reactivity of AIH-1 sera towards citrullinated antigens in the anti-CCP test appeared to be highly polyclonal, and often is not dependent on the citrulline-moiety. For some AIH-1 sera the presence of arginines or positively charged residues in the antigen enhances the reactivity, but is not an absolute requirement.
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064 DIAGNOSTIC VALUE OF ANTI-CYCLIC CITRULLINATED PEPTIDE ANTIBODIES IN PATIENTS WITH RHEUMATOID ARTHRITIS TREATED WITH ETANERCEPT

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Anti-CCP antibodies are highly specific to rheumatoid arthritis (RA) compared to rheumatoid factor and show a reasonable sensitivity. Furthermore this newly identified class of antibody appears to be a good discriminating marker between erosive and nonerosive RA. There is a controversy whether CCP antibody levels respond to TNF blocking therapy by infliximab. No data exist on CCP antibody levels in the course of an anti-TNF therapy by etanercept.

It was the aim of the study to monitor changes in anti-CCP antibody levels in the course of anti-TNF (etanercept) treatment of RA patients and to evaluate the importance of this antibody in association to the presence of the SE as a predictive marker for therapy outcome.

Serum samples were obtained from 86 RA patients (mean age 51.4+2.1 years, 73 female, 13 male) treated with etanercept (2×25 mg/week) prior to treatment and, in 48 cases, 3-4 months after the first dose of etanercept. Disease activity and therapy response were assessed according to http://www.das-score.nl. All patients were characterised by a high disease activity at the start of treatment (ESR 57+5 mm/h, TJC 10.2+0.8, SJC 10.0+0.7, DAS28 6.2+0.2, mean+SEM). Serum levels of anti-CCP antibodies were analysed by ELISA (Immunoscan RA Mark 2, Euro-Diagnostika, the Netherlands) according to the manufacturer's instructions. For exclusion of false negative results two additional assays were used.

At baseline, anti-CCP antibodies were found to be positive in 64 out of 86 patients (81.1%), in 19 patients (22.1%) the CCP-antibody levels were extremely high and exceeded 1600 U/ml. Within 3–4 months of etanercept treatment antibody levels did not change significantly (all:

1035+199 U/ml before treatment, 1068+197 U/ml 3–4 months later, mean+SEM). At baseline, patients with a good clinical response to etanercept differed from the non-responder group regarding their anti-CCP antibody levels, with means of 546+170 U/ml and 1898+471 U/ml, respectively. For selected patients anti-CCP levels were tested in a up to four year follow-up. The course of antibody levels was not associated with the disease activity score.

The lack of anti-CCP antibodies in a selected group of highly active, therapy refractory RA patients with a longstanding disease and with radiological joint changes shows the limitations of the anti-CCP antibody determination.

In contrast to reports describing a decrease of anti-CCP levels in RA patients showing clinical improvement following infliximab therapy in our study no decrease of anti-CCP antibody levels was seen, this includes the group of good therapy responders. However, our preliminary results suggest that the antibody status at the start of an anti-TNF therapy is associated with the clinical outcome.

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065 DEIMINATED EPSTEIN BARR VIRUS NUCLEAR ANTIGEN I IS A TARGET OF ANTI-CITRULLINATED PROTEIN ANTIBODIES IN RHEUMATOID ARTHRITIS

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A number of rheumatoid arthritis (RA) sera contain antibodies specific for peptides in which arginine is substituted by the deiminated form citrulline (anti-citrullinated peptide antibodies, ACPA). These antibodies represent a marker of RA in the early phases of the disease.

A comparative evaluation of sequences recognised by ACPA shows that a critical feature is the presence of citrulline flanked by neutral aminoacids such as glycine, serine or threonin. Similar aminoacid repeats are commonly found in viral proteins.

EBNA I, one of the nuclear proteins encoded by Epstein-Barr virus, whose relationship with RA is still being debated, is characterized by a region (aa 35–58) containing a six fold Arg-Gly repeat. We tested the hypothesis that deimination of viral sequences containing Arg-Gly repeats could generate epitopes recognized by ACPA.

Antibodies specific for a peptide corresponding to the EBNA I 35–58 sequence containing citrulline in place of arginine (Viral Citrullinated Peptide) were detected in 50% of RA sera and in less than 5% of normal and disease control sera. In addition, affinity purified anti-VCP antibodies from RA sera reacted with filaggrin-derived citrullinated peptides and with deiminated fibrinogen and with deiminated recombinant EBNA I.

Moreover, anti-VCP antibodies immunoprecipitated, from the lysate of calcium ionophore-stimulated lymphoblastoid cell lines, an 80 kDa band that was reactive with a monoclonal anti-EBNA I antibody and with anti-modified-citrulline antibodies.These data indicate that ACPA react with a viral deiminated protein and suggest that EBV infection may play a role in the induction of these RA-specific antibodies.

066 HUMAN ANTIBODIES AND THEIR ROLE IN ANIMAL MODEL OF AUTOIMMUNE ARTHRITIS

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The role of antibodies in the pathogenesis of rheumatoid arthritis (RA) has attracted significant attention despite the longstanding controversy as to whether such antibodies contribute to, or are secondary to, the pathogenesis of RA. In addition to rheumatoid factor (RF) present in 60–80% of RA patients, antibodies of a more specific nature recognising citrullinated residues in polypeptides are now associated with the onset of the disease. However, currently it is unclear whether human IgG autoantibodies are simply a marker for or are directly involved in the pathogenesis of RA.

To address the potential pathogenicity of human RA-associated antibodies, we developed a passive transfer model involving mice deficient in the low affinity inhibitory Fc receptor, FcgRIIB. We report that blood samples (plasma or serum) from patients with established RA (n = 4), but not healthy non-RA individuals (n = 4), can induce joint inflammation and histological lesions in FcgRIIB knockout mice consistent with underlying RA syndrome. FcgRIIB deficiency is required for mice to become susceptible to arthritis induced by human blood samples. Importantly, our data show that the pathogenic component of the human serum leading to induction of arthritis in mice resides in the immunoglobulin fraction. It remains to be demonstrated whether antibodies recognising cyclic citrullinated peptide (anti-CCP), or other undefined autoantibodies, are responsible for the inflammatory lesions we observed.

In summary, these results provide evidence that humoral autoimmunity can contribute directly to autoimmune arthritis, and that FcgRIIB knockout mice are a promising model to evaluate the arthritogenic potential of human autoantibodies. Moreover, this opens a new way to elucidate the therapeutic potential of anti-rheumatic drugs targeting different factors.

067 LYMPHOID CHEMOKINES CXCL13 AND CCL21 ARE ABNORMALY UPREGULATED IN SALIVARY GLANDS OF MALT LYMPHOMAS IN SJÖGREN'S SYNDROME AND ASSOCIATE WITH THE HISTOLOGICAL ORGANISATION OF THE REACTIVE AND MALIGNANT COMPONENTS OF THE LYMPHOID PROLIFERATION

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Background: We previously demonstrated that CXCL13 and CCL21 expression in Sjögren's syndrome (SS)-minor salivary glands (SG) was associated with the acquisition of lymphoid organisation by the inflammatory foci. A role for these reactive structures in the lymphomatous transformation in SS has been suggested, though the role of lymphoid CKs in this process has not been elucidated.
Materials and Methods: 21 parotid biopsies of patients with SS and MALT lymphoma (MALT-L) and 3 biopsies of SS- MSG before the development of MALT–L have been studied. To define the lymphoid infiltrate organisation (reactive vs. malignant areas) and identify the B cell subpopulations, stainings for cellular and vascular markers have been performed (CD21, bcl-2, bcl-6, IgD, CD20 e CD3, CD68, CD31 e PNAd) on sequential sections. Electronic pictures of CXCL13 and CCL21 were analysed by thresholding the colour of positive staining in histologically detected areas of interest. The system estimated the volume fraction areas of the CKs : ratio between the area of malignant v.s reactive components/area positive for CXCL13 or CCL21. CC13 and CCL21 producing cells were identified by double stainings for different cell markers.
Results: In 100% of MALT-L samples, reactive areas characterised by T/B cell segregation, CD20+IgD+bcl-2+/follicular B-cells, Germinal Centers and high endothelial venules formation were detected. A B cell population lacking IgD, characterised by nuclear atypies was consistently observed within the ductal proliferation and identified as malignant. Increased expression of CXCL13 and CCL21 was observed in 100% of MALT samples (MALT-L vs. MSG respectively $P<0,05$ and $P<0,0001$). High density of CXCL13+ cells was observed within the reactive areas and surrounding lympho-epithelial islands; while a little expression of CXCL13 was detected within the malignant areas and inside the ducts. Significant difference between the expression of CXCL13 in the reactive areas compared to the lymphomatous infiltrated areas was estimated ($P<0.001$). CCL21 expression was mainly detected within the T cell area of the reactive component in both MSG and MALT-L.

The double stainig performed showed CXCL13 expression in a small population of CD3+ and CD68+ cells , while no CCL21 expression was observed in CD68, CD3 or CD20+ cells. We did not detect any clear CK production by the malignant component.
Conclusions: The aberrant expression of CXCL13 and CCL21 within reactive and neoplastic ares in the MALT-L suggest a functional role for these molecules in the organisation and maintenance of the histological structures (GCs and LESA) that appear to be support the immune process and malignant progression.

068 PROPORTIONS OF INFILTRATING SYNOVIAL T-LYMPHOCYTES ARE ASSOCIATED WITH ELEVATED PLASMA CRP LEVELS IN OSTEOARTHRITIS

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Objectives: Synovial membrane mononuclear cell infiltrates containing macrophages and T-lymphocytes have been described in patients with osteoarthritis (OA). Although the macrophages within these infiltrates have been the subject of many studies, the nature and function of the synovial T-cells are unclear. We assessed the expression of CD4 and CD8 by synovial T-cells in OA, and investigated whether synovial T-cell subsets are associated with CRP elevations seen in these patients.

Methods: PB and SM specimens from patients with OA or RA were obtained at the time of arthroscopy or total joint arthroplasty. PBMCs and SM cells were isolated and subjected to flow cytometric analysis using fluorescently-labeled anti-CD3, anti-CD4 and anti-CD8 monoclonal antibodies. High-sensitivity (hs)CRP levels in plasma were measured by ELISA.
Results: CD4+ cells outnumbered CD8+ cells in the SM of 8 of 10 patients with OA and all patients with RA. Proportions of CD4+ cells, but not CD8+ cells, within the SM of patients with OA correlated with hsCRP levels ($r=0.73$; $p=0.01$). In addition, there was enrichment of $CD4^-CD8^-$ (SM: $15.95\%\pm1.80$, PB: $5.35\%\pm0.62$, $p<0.0001$), and $CD4^+CD8^+$ cells (SM: $3.80\%\pm1.13$, PB: $1.89\%\pm0.46$, $p=0.049$), in SM from OA patients. Smaller increases in the double-negative and double-positive subsets were seen in RA SM.
Conclusions: In the majority of OA patients, CD4+ cells outnumber CD8+ cells within the SM, and CD4+ cells in SM correlate with hsCRP levels measured in peripheral blood. Furthermore, double-negative and double-positive T-cell subsets were enriched in the SM of OA patients compared with peripheral blood. $CD4^-CD8^-$ cells in the SM of patients with OA may represent NKT-cells, γδT-cells, premature $CD3^+$ cells generated through a process of extra-thymic maturation, or previously activated single-positive T-cells that have lost cell-surface expression of CD4 or CD8. Further characterisation of these SM T-cells and their function is likely to advance our understanding of OA pathogenesis.

069 OVEREXPRESSION OF THE CALCIUM-CALCINEURIN-NFAT SIGNALING PATHWAY IN RHEUMATOID ARTHRITIS SYNOVIUM

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Objectives: To determine the spatial expression in synovium of the calcineurin B subunit (CnB), cyclophilin A (CycA), FK506 binding protein (FKBP12), and the calcineurin-regulated transcription factors nuclear factor of activated T cells (NFAT) 1–4. To quantify differences in CnB and NFAT 1–4 expression between inflamed and non-inflamed synovia.
Methods: Tissues used: Synovial biopsies or surgical specimens obtained during arthroplasty from patients with RA with active disease despite DMARD treatment (n=8–10), failed total knee arthroplasty (n=7), and OA (n=8–11). CnB expression was also determined in control synovia obtained during surgery for avascular necrosis of the femur or meniscus surgery (n=13). The spatial expression of all factors was determined by immunohistochemistry with non-specific isotypes as negative controls. Expression differences were quantified by counting the number of CnB or NFAT-expressing cells per high-power field (400×) separately in the surface layer of the synovial lining, the full thickness of the lining, or the subintima. Statistical significance was determined by ANOVA and the Mann-Whitney U Test.
Results: There was widespread expression of all factors in the lining and subintima of all inflamed specimens. In particular, the expression patterns of CnB, CycA and FKBP12 resembled each other closely, indicating the presence of CnB and its co-regulators in the same cells. In the surface layer of the lining, there were no significant differences in expression of CnB or NFAT 1–4. However, when the numbers of CnB or NFAT 1–4 expressing cells were determined for the entire thickness of the lining, all five factors were expressed between four to six fold more frequently in RA than in OA ($p<0.05$). Remarkable statistically significant differences among the arthropathies were also detected in the subintima: CnB and all four NFATs were expressed most in RA, less in failed arthroplasty, and least in OA (5–25% of the expression in RA, depending on the factor). Expression in 13 surgical control specimens was determined for CnB and was 34.5% (+/− 11.3%) lower than in OA, but this difference was only marginally significant. The differences in subintimal expression among the arthropathies were mostly due to the higher expression in RA in inflammatory infiltrates—notably in CD68+ macrophages, followed by lymphocytes and plasma cells—and also vascular endothelium.
Conclusions: The major players in the Ca++-calcineurin-NFAT signaling pathway were detected in several synovial cell types important in the pathogenesis of inflammatory arthropathies and were found to be over-expressed in RA. These findings suggest that Ca++-calcineurin-NFAT signaling may play roles in multiple steps in the pathogenesis of inflammatory arthropathies. They suggest that calcineurin inhibitors such as CsA may function in the treatment of RA in part by interfering with Cn signaling in the synovium and underscore the potential efficacy of novel NFAT-directed pharmacologic agents as DMARDs.

070 SPONTANEOUS CARDIAC VALVE INFLAMMATION IN THE K/BXN MURINE ARTHRITIS MODEL

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Aim: Valvular endocarditis complicates several human rheumatologic conditions, including acute rheumatic fever, systemic lupus erythematosus, and rheumatoid arthritis, highlighting the systemic nature of these inflammatory diseases. The pathogenic mediators of valvulitis remain poorly understood, and few animal models exist. K/BxN T-cell receptor (TCR) transgenic mice develop arthritis due to T and B cell cooperation, resulting in the production of pathogenic autoantibodies against the ubiquitously-expressed antigen glucose-6-phosphate isomerase. We investigated whether the heart was a target of immune attack in the K/BxN transgenic mouse model of arthritis.
Methods: Standard histochemical and immunohistochemical techniques were employed to study sections of hearts obtained from arthritic K/BxN transgenic mice and genetically-related strains. Additionally, the influence of the complement component C1q was studied by utilising K/BxN mice lacking the C1q gene.
Results: We observed inflammation of cardiac valves in 35 of 36 (97.2%) K/BxN arthritic mice. The primarily mononuclear inflammation was restricted to the left-sided cardiac valves (mitral and aortic valves), and was accompanied by valve thickening and proliferation of elastin filaments. Valvulitis was not observed in non-arthritic, transgene-negative (BxN) littermates (0 of 15 mice). Similarly, non-arthritic mice bearing the KRN transgene in the absence of any genetic contribution from the NOD background (KRN/B6) did not develop valvulitis (0/3 mice). As in the case of arthritis, the key contribution from the NOD background is the MHC class II allele $I\text{-}A^{g7}$, since 15 of 15 congenic KRN/B6.g7 arthritic mice developed valvulitis. Genetic absence of C1q resulted in more severe valve inflammation in this model. In contrast to arthritis, we have been unable to induce valvulitis by transfer of serum from affected K/BxN mice to normal recipient mice.
Conclusions: We have described a new animal model of spontaneous cardiac valvulitis, occurring in the K/BxN mouse model of arthritis. As is the case for arthritis in this model, the initiation of valve inflammation depends on the presence of the KRN TCR transgene and the MHC class II restriction element $I\text{-}A^{g7}$. However, different effector mechanisms or tempos of disease induction may be at play in arthritis versus valvulitis, since transfer of arthritogenic K/BxN serum does not cause valvulitis and since C1q deficiency results in more aggressive valvulitis without influencing arthritis severity. Our observations broaden the scope of target organs in the K/BxN arthritis model to include the heart, emphasizing the systemic nature of the autoimmune process in this model and the model's relevance to systemic human arthritidies. Furthermore, this model opens new avenues toward understanding the pathogenesis of cardiac valvulitis

071 SYNOVIAL FLUID FROM RHEUMATOID ARTHRITIS PATIENTS STIMULATES CCL18 SECRETION, WHICH IS INDEPENDENT OF IL-10 AND IL-13

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Background: Chemokines are critical in the orchestration of leucocyte attraction. CCL18 is a chemokine that is produced by macrophages and dendritic cells and is known to attract naïve T cells. The regulation of CCL18 is complex and it remains controversial which immune cells and what stimuli are responsible for CCL18 production in RA
Objective: To investigate what factors regulate the secretion of CCL18 in normal individuals and in rheumatoid arthritis.
Methods: Sections of synovial tissue of RA patients and controls were stained for CCL18 expression. Monocyte derived dendritic cells were cultured with IL-4 or IL-13 in combination with GM-CSF and stimulated with IL-1, IL-10, IL-13, IL-15, IL-17, IL-18, IFN-g, CD40L, RANKL or with Toll-like receptor (TLR) agonists pam3cys, poly (i:c), LPS or R848. In addition, monocytes were cultured and stimulated with combinations of IL-4, IL-13 and IL-10. Finally, the regulation of CCL18 production by synovial fluid was examined. CCL18 production was measured with ELISA techniques.
Results: A high CCL18 expression was detected in RA synovial tissue, whereas only a minor CCL18 expression could be detected in control synovial tissue. In contrast to various pro-inflammatory stimuli or TLR driven maturation, IL-10 strongly stimulated monocyte derived DC to secrete CCL18. On monocytes, IL-10 only induced a minor CCL18 production, whereas IL-4 and IL-13 were more potent CCL18 inducers. Interestingly, IL-10 induced high amounts of CCL18 in combination with IL-4 or IL-13 when administered to monocytes. Incubation with synovial fluid from RA patients also resulted in a marked CCL18 production and

clearly enhanced the effects of IL-4, IL-13 and IL-10. Blockade of IL-10 and/or IL-13 in synovial fluid with antibodies did not inhibit this effect of synovial fluid. Intriguingly, the incubation with synovial fluid resulted in an imprinting phenomenon, since the effects on CCL18 secretion persisted when the synovial fluid was no longer present.
Conclusions: Here we demonstrate that IL-4 and IL-13 are critical regulators of CCL18 production on monocyte derived cells and strongly synergise with IL-10. Cells that have been in contact with synovial fluid also have a synergistic CCL18 secretion upon stimulation with IL-4, IL-13 and IL-10. Surprisingly, this is not driven by IL-10 of IL-13, suggesting the presence of another CCL18 inducing factor in synovial fluid. Our findings might partly explain the enhanced local CCL18 expression in RA synovial tissue.

072 EXPANSION OF TLR-9 BUT NOT TLR-2, -3, AND -4 EXPRESSING B-CELLS AND MONOCYTES IN ACTIVE SYSTEMIC LUPUS ERYTHEMATOSUS

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Objective: Toll-like receptors (TLRs) are pattern associated receptors of innate immunity that may be involved in the recognition of self antigens, induction and/or amplification of autoimmune responses in systemic lupus erythematosus (SLE). We examined the expression of various TLRs on subpopulations of peripheral blood mononuclear cells (PBMCs) of SLE patients and their function.
Patients and Methods: PBMCs from 50 SLE patients with active (SLE disease activity index (SLEDAI) $\geqslant$8, n=26) and inactive disease (SLEDAI$<$8, n=24) and 20 healthy controls were studied for the expression of TLRs by flow cytometric analysis. TLR expression was evaluated on various subpopulations of PBMCs (TLR-2 and TLR-4 by membrane staining, whereas expression of TLR-3 and TLR-9 intracellularly). Toll-like receptor function was accessed by stimulating PBMCs with specific ligands.
Results: We found an increased proportion of B-cells and monocytes that expressed TLR-9 among patients with active (49.5%$\pm$24.4% and 30.7%$\pm$24.1% respectively) compared to patients with inactive disease (22.8%$\pm$19.6%, p=0.02 and 14.3%$\pm$8.4%, p=0.029 respectively). Interestingly, a decreased proportion of CD4+CD25++ cells was found to be positive for the expression of TLR-9 among patients compared to healthy controls but this did not reach statistical significance (48.34$\pm$23.32 versus 70.40$\pm$28.13, p=0.24). Increased expression of TLR-9 in SLE patients was associated with enhanced response to TLR-9 ligands demonstrated by an enhanced proliferative response and higher IL-6 and IL-10 production in patients with active disease not receiving hydroxychloroquine, an inhibitor of TLR-9 signaling. Among B-cells, increased proportions of plasma-cells and memory B-cells, but not naïve B-cells expressed TLR-9 in patients with active SLE. Increased percentages of TLR-9 expressing B-cells correlated with the presence of anti-dsDNA antibodies (p=0.007). An enrichment in the TLR-9 positive plasma-cell population was detected in the bone marrow compared to peripheral blood in active SLE who received no cytotoxic treatment. While SLE serum induced an increase in the percentage of TLR-9 expressing plasmacells from healthy donors, normal serum had no effect.
Conclusions: An increased proportion of monocytes, memory B-cells and peripheral plasma-cells express TLR-9 in patients with active SLE compared to patients with inactive disease. Endogenous nucleic acid released during apoptotic cell death in SLE patients may stimulate B-cells towards autoantibody production and monocytes towards differentiation to dendritic cells via these receptors, thus contributing to disease pathogenesis.

Molecular pathways and regulation of inflammation/ signalling

073 WEGENER'S AUTOANTIGEN INDUCES MATURATION OF DENDRITIC CELLS AND LICENCES THEM FOR TH1 PRIMING: INVOLVEMENT OF THE PROTEASE ACTIVATED RECEPTOR-2 PATHWAY

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Autoantibodies to proteinase 3 (PR3) are involved in the pathogenesis of autoimmune-mediated vasculitis in Wegener's granulomatosis (WG). To address the question how a particular autoantigen – PR3 – becomes target of adaptive immunity, we investigated the effect of PR3 on immature dendritic cells (iDCs) in normal donors and WG and Crohn's disease (CD), and by which mechanism this effect was induced. PR3 induces phenotypic and functional maturation of blood monocyte-derived iDCs. PR3-treated DCs express high levels of CD83, a DC-restricted marker of maturation, CD80 and CD86, and HLA-DR. Furthermore, they become fully competent antigen presenting cells and can therefore induce stimulation of PR3-specific CD4+ T cells, which produce IFN-γ. PR3-maturated DCs derived from WG patients induce a higher response of PR3-specific CD4+ T cells as compared to CD patients and normal donors. The maturation of DC mediated through PR3 was inhibited by a serine protease inhibitor, by antibodies directed against the protease-activated receptor-2 (PAR-2) and by inhibition of phospholipase C. These results suggest that the interactions of PR3 with PAR-2 are possible involved in the induction of DC maturation. Wegener's autoantigen interacts with a ''gateway'' receptor (PAR-2) on iDCs in vitro triggering their maturation and licences them for a Th1-type response potentially favouring granuloma-formation in WG.

074 CITRULLINATION IS AN INFLAMMATION DEPENDENT PROCESS

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Background: The presence of citrullinated proteins was initially considered specific for the RA synovium. However, recent reports demonstrated that citrullinated proteins are also present in non-RA synovitis. Moreover, citrullination occurs in autoimmune neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease. In the light of these observations we asked the question of whether citrullination occurs in other types of inflammation than arthritis, and to which extent citrullination is present in the normal joint.
Methods: We obtained synovial tissue from 19 RA patients and 10 healthy individuals, muscle tissue from 6 polymyositis (PM) patients and 7 control individuals, intestinal tissue from 10 inflammatory bowel disease (IBD) patients from both macroscopically affected and non-affected areas and tonsil tissue from 4 patients with chronic tonsil inflammation. The presence of citrullinated proteins was immunohistochemically detected with a rabbit polyclonal antibody against modified citrulline residues. Biopsies were evaluated by double blind semi-quantitative analysis. Statistical analysis was performed using the Mann-Whitney test for histological scores and Fisher's exact test for proportions.
Results: Citrullinated proteins were present in all RA samples, while only 30% of the healthy synovial samples were positive with scarce amounts of citrullination ($p<0.05$). Citrullination was identified both in the lining and the sublining layers, intracellular as well as extracellular. Endothelial cells, fibroblasts and mononuclear cells were positive for citrullinated proteins while extracellular citrullination was mainly located to the amorphous fibrin areas. All PM samples were positive ($p<0.05$) with citrullinated proteins present both in endothelial and inflammatory cells, while muscle fibers were negative. Control muscle samples were negative. Even though more frequently detected in the macroscopically affected colonic areas (70%) as compared to the macroscopically non affected colonic areas (30%, $p>0.05$) of the IBD patients, no significant difference in the pattern or extend of citrullination was observed between the two entities. Citrullination was present mainly in the lamina propria cells but also in the epithelial cells of Lieberkuhn's crypts. All tonsil samples were positive with citrullinated proteins present both in the T cell as well as B cell areas.
Conclusion: Our findings demonstrate that citrullination is not a disease-specific but a more general phenomenon in the context of inflammation.

075 TLR4 INDUCED FCγ RECEPTOR EXPRESSION POTENTIATES EARLY ONSET OF JOINT INFLAMMATION AND CARTILAGE DESTRUCTION DURING IMMUNE COMPLEX ARTHRITIS: COUPLING OF INNATE TO ADAPTIVE IMMUNITY

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Background: In earlier studies we have found that FcγR expression on synovial macrophages drive the onset of murine immune complex mediated arthritis (ICA).[1] However, in a normal joint, FcγR expression is low. Other receptor families, like toll-like receptors (TLR) may be involved

in first recognition of IC and their activation may thereby potentiate FcγR expression driving the onset of arthritis.
Aim: To study the role of TLR4 in joint inflammation and cartilage destruction during ICA and its relation with FcγR expression.
Results: ICA was passively induced in knee joints of TLR4−/− mice (C3H/hey) and their wildtype controls (C3H/hen) by injection of anti-lysozyme intravenously prior to intra-articular injection of PLL coupled lysozyme. At day 1 after ICA induction, joint swelling as measured by 99mTc uptake, was 56% lower in knee joints of TLR4−/− mice when compared to WT controls. The amount of inflammatory cells in synovium (infiltrate) and joint space (exudate) was inhibited by 79% and 68% respectively. As ICA is completely FcγR regulated, we additionally measured mRNA expression of FcγR in arthritic synovia using quantitative RT-PCR. TLR4 was largely responsible for potentiation of all three FcγR (FcγRI, II and III 46%, 56% and 70% respectively). Whether upregulation of FcγR resulted in enhanced cytokine production was measured in washouts of synovial specimen using Bioplex. IL-1, IL-6, MIP-1a and KC production was significantly potentiated by TLR4 with respectively 49%,72%,68% and 84%. Whether upregulation of FcγR and cytokines by TLR4 had consequences for cartilage destruction was further investigated by measuring PG depletion in total knee joints using image analysis. TLR4 potentiated early PG depletion by 80%. However at day 4 after ICA induction, joint inflammation and cartilage destruction (proteoglycan depletion, MMP-mediated cartilage matrix destruction and chondrocyte death) was completely comparable between TLR4−/− mice and their WT controls indicating that TLR4 is only important in the first phase of this arthritis.
Conclusion: TLR 4 regulates early onset of joint inflammation and cartilage destruction during immune complex mediated arthritis by potentiation of FcgR expression and enhanced cytokine production.

1. **Nabbe KC**, Blom AB, Holthuysen AE, *et al*. Coordinate expression of activating FcgR I and III and inhibiting FcgR II in the determination of joint inflammation and cartilage destruction during immune complex-mediated arthritis. *Arthritis Rheum* 2003;**48**:255–65.

076 ACTIVATION OF PI3 KINASES DOWNREGULATE IL-1 β BUT TRIGGER SIL-1RA PRODUCTION IN HUMAN MONOCYTES

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The unbalanced production of IL-1 β and its secreted inhibitor sIL-1Ra plays an important role in chronic inflammation. Relevant to this condition, direct cellular contact with stimulated T cells is a potent inducer of cytokine production in human monocytes/macrophages. We previously demonstrated that activation of PI3 kinases (PI3Ks) controls the transcription of sIL-1Ra gene in IFN β-activated monocytes. In this study we addressed the question of PI3K involvement in the production of IL-1 β and sIL-1Ra in monocytes activated by cellular contact with stimulated T cells (mimicked by CHAPS-solubilised membranes of stimulated T cells, CEsHUT), or LPS, in order to compare stimuli involved in conditions relevant to chronic and acute inflammation, respectively. In monocytes activated by either CEsHUT or LPS, the PI3Ks inhibitor, Ly294002 reverted sIL-1Ra transcript expression and sIL-1Ra production to basal level. This demonstrates that PI3Ks controlled the induction of sIL-1Ra gene transcription. In contrast, Ly294002 increased the production of IL-1 β protein, in both CEsHUT- and LPS-activated monocytes, the enhancement being drastically higher in the former. This was not due to changes in IL-1 β mRNA steady-state levels and transcript stability as demonstrated by using actinomycin D in time-course experiments. In addition, the determination of intracellular and secreted IL-1 β levels suggests that PI3Ks were mainly implicated in the down-regulation of IL-1 β secretion when cells were activated by LPS, whereas in CEsHUT-activated monocytes PI3Ks repressed both IL-1 β protein secretion and yet-undefined mechanism. Interestingly, while IL-1 β transcripts induced by CEsHUT displayed a higher degree of instability after 3 h activation (i.e., when steady-state was reached) than those induced by LPS, sIL-1Ra transcript half-life was shorter in LPS-activated monocytes than in CEsHUT-activated cells. Together, the present results demonstrate that PI3Ks are involved in the repression of IL-1 β and the induction of sIL-1Ra production in isolated human blood monocytes by controlling different mechanisms in conditions mimicking chronic/sterile (CEsHUT) and acute/infectious (LPS) inflammation. Thus PI3Ks represent key effectors that might be uncontrolled in pathological conditions. This further suggests that the stimulation of PI3K pathways may be an effective approach for preventing or treating chronic/sterile or dysregulated acute inflammation.

077 ROLE OF MYD88 DEPENDENT TOLL-LIKE RECEPTOR SIGNALING PATHWAY IN WOUND HEALING AND SCLERODERMA

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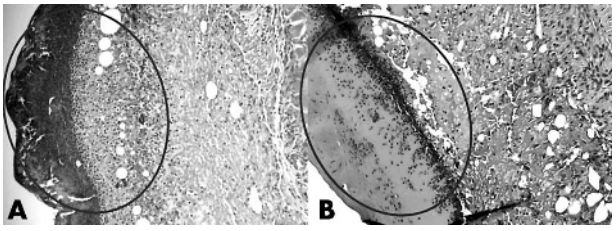
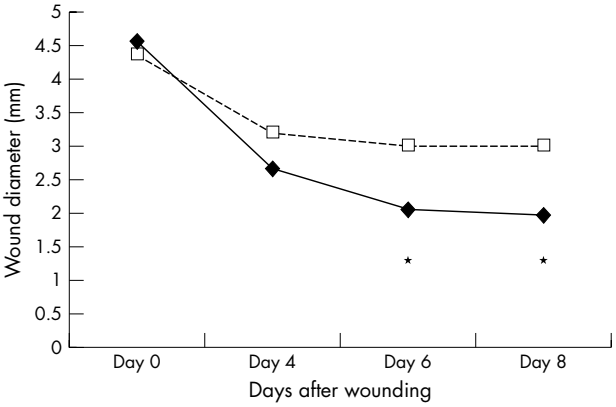
Aim: Wound healing is utilised in our study as a model for systemic sclerosis (SSc) based on their shared features of cell-matrix-cytokine interactions that proceed from inflammation to tissue remodeling and fibrosis. Toll-like receptors (TLRs) have been shown to play critical roles in regulation of the innate immune system and defense against microbes. Certain components of extracellular matrix (ECM) have been shown to stimulate endothelial recognition of injury through TLR as their endogenous ligands. MyD88 is a key adapter protein for TLR common signaling pathway. In order to understand how the innate immune system affects dermal ECM remodeling in SSc, we studied wound healing in mice deleted of MyD88.
Methods and Materials: MyD88 −/− and MyD88 +/− mice were used. Four full thickness wounds were produced on the back of each animal using a 5 mm circular punch biopsy. Wound diameter of all 4 wounds/mouse was measured at the time of wounding and every two days after wounding until day 8. At day four, six and eight the mice were sacrificed and circular regions of the wound and surrounding tissue were collected using 9 mm circular punch biopsy blades. Sections were embedded in paraffin, processed for H&E and immunohistochemistry (SMA – smooth muscle actin) staining, and compared by light microscopy.
Results: We have observed: 1) significantly faster wound healing in MyD88 +/− mice compared to MyD88 −/− mice ($p<0.05$ at day 6 and day 8), see fig2; 2) less inflammatory infiltrate and granulation tissue in MyD88 −/− mice (see fig1); and 3) no apparent difference in myofibroblast distribution between MyD88 −/− and +/− animals (SMA stain).
Conclusions: We propose the above results suggest that MyD88-dependent TLR signaling plays a crucial role in normal wound healing and tissue remodeling, likely through endogenous TLR ligand(s). The finding of no apparent effect of MyD88 deletion on myofibroblasts in wounds is intriguing. It is plausible that TLRs are not part of TGFβ



Abstract 077 Figure 1 Histopathology of healing wounds from control (A) MyD88 +/− mouse and from MyD88 −/− mouse (B) 6 days after creating full thickness excisional wounds. Ovals indicate the healing wound margin/eschar. Dermal ECM tissue is towards right in both panels A and B.



Abstract 077 Figure 2 Rate of wound closure in MyD88 −/− (■) compared to MyD88 +/− control mice (♦). Points shown are average values of 4 wounds/animal/day. Differences between day 6 and day 8 wounds are statistically different at $p<0.05$ (students t-test).

signaling or that alternative TLR adaptor proteins mediate TLR signaling for TGFβ production. These results may help elucidate new roles of TLRs in SSc and thus suggest new approaches to therapy.

078 INVOLVEMENT OF TUMOR NECROSIS FACTOR - ALPHA IN THE CONSTITUTIVE OR THE LPS-INDUCED TOLL-LIKE RECEPTOR 4 EXPRESSION

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Introduction: Stimulation of the toll-like receptor 4 (TLR-4) by lipopolysaccharide (LPS) results, among others, to the secretion of tumour necrosis factor-α (TNFα) and to the upregulation of the surface expression of ICAM.1 adhesion molecules and of TLRs. However, the effect of TNFα in the expression of TLR4 and to the LPS-induced apoptosis remains unclear. To investigate this, we examined the effect of exogenous TNFα addition, as well as of TNFα blockade, in resting or LPS-challenged Bone Marrow Mononuclear Cells (BMMC) and in neoplastic monocytic THP-1 cells.
Materials and Methods: BMMC derived from 5 normal individuals and 8 patients with myelodysplastic syndromes were included in the study. All the cell lines studied were treated for 12, 24 and 48 hours, with LPS (1μg/ml), TNFα (200-IU/ml), anti-TNFα (infliximab; 10 μg/ml) and with concurrent LPS and TNFα blockage. TLR-4 mRNA levels were assessed by quantitative Real-time PCR, whereas TLR-4 and ICAM.1 protein expression was examined by flow cytometry. Apoptosis was monitored by Annexin-V binding assay.
Results: TNFα treatment resulted to a 70.0±2.0% increase of the TLR-4 protein expression in BMMC, but not in THP-1 cells, whereas significantly upregulated ICAM.1 expression was assessed in both populations (20-fold increase). In all types of cells, LPS treatment led to TLR-4 induction at both the mRNA (two fold) and protein level (5 and 1.5 fold in BMMC and THP-1, respectively). It also strongly induced ICAM.1 expression in both BMMC and THP-1 cells (80-fold and 14-fold, respectively). TNFα blockade completely inhibited the TLR-4 protein expression in resting and in LPS-stimulated BMMC and THP.1 cells, whereas an 80% reduction was observed at the mRNA level. Regarding ICAM.1, anti-TNFα treatment had no effect in the constitutive, but decreased the LPS-induced expression to half.

Apoptosis increased after TNFα treatment or LPS stimulation in both BMMC and THP-1 (resting: 10.0±2.7% and 12.2±0.8%, TNFα: 19.0±1.3% and 16.0±0.7%, LPS: 27.0±2.9% and 42±1.4% respectively). Anti-TNFα treatment reduced apoptosis in both resting and LPS-treated cells. Double staining revealed that 73.0±6% of Annexin-V+ cells were TLR-4+, while 83.0±6% of TLR-4+ cells were Annexin-V positive in untreated BMMC. After LPS challenge 91.1±4.0% of Annexin-V+ cells were TLR-4+ whereas 46.0±6.8% % of TLR-4+ cells were Annexin-V+.
Conclusion: LPS stimulation leads to strong induction of TLR-4 and ICAM.1 expression as well as of apoptotic cell death. Our data show that LPS effect on TLR-4 levels, and partially in apoptosis, is TNFα mediated. Moreover, the constitutive expression of TLR4 is also TNFα dependent. In contrast, TNFα is only partially implicated in LPS-induced ICAM.1 expression.

079 POLY-INOSINIC:CYTIDYLIC ACID (POLYI:C) INDUCES SIGNIFICANT FAS INDEPENDENT APOPTOSIS IN CULTURED NON-NEOPLASTIC SALIVARY GLAND EPITHELIAL CELLS

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Aim: Primary Sjögren syndrome (SS) is characterised by dysfunction, as well as destruction of salivary gland epithelial cells associated with programmed cell death or apoptosis. Reported evidence from this laboratory indicates that cultured non-neoplastic salivary gland epithelial cells (SGEC) from patients with SS are intrinsically activated and express constitutively high levels of surface Fas protein. In a manner similar to other types of cells, we have previously demonstrated that cultured SGEC pretreated with a protein or RNA synthesis inhibitor undergo significant apoptosis following triggering of surface Fas molecules. Toll-like receptors are transmembrane receptors involved in the recognition of foreign pathogen-associated molecular patterns and activation of processes that lead to innate immune responses. Recent evidence had indicated that TLR signalling also leads to the activation of apoptotic mechanisms in several types of cells, thus possibly regulating the inflammatory responses. In this study we investigated whether triggering of TLR-2, -3 and -4 by native or synthetic ligands in cultured SGEC can induce apoptosis and the apoptosis-related molecules Fas(CD95) and Fas-ligand (CD178).
Materials and Methods: Cultured, non-neoplastic SGEC were stimulated by peptidoglycan (PGN; TLR2-ligand; 100 μg/ml), poly-inosinic: cytidylic acid (polyI:C; TLR3-ligand; 5 μg/ml) and lipopolysaccharide (LPS; TLR4-ligand; 1 μg/ml) for 24 and 48 hours with or without prior treatment (1 hour) with the RNA synthesis inhibitor actinomycin D (ActD, 0.5 μg/ml). The induction of apoptosis was monitored by the Annexin V-binding assay. As control, SGEC pretreated with ActD were also stimulated by the anti-Fas monoclonal antibody (mAb) CH-11. The neutralising anti-Fas mAb DX2 was also applied to block the induction of Fas-mediated apoptosis. Treated and untreated cells with TLR-ligands were also examined for the expression of CD95/Fas and CD178/FasL by flow cytometry.
Results: Stimulation of SGEC by polyI:C (but not PGN or LPS) resulted in a significant increase of Fas expression on SGEC (30% increase after 48hr stimulation) compared to untreated cells. The stimulation of cultured SGEC by TLR-ligands or CH-11 alone did not result in the induction of apoptosis. Stimulation of ActD-pretreated SGEC by CH-11 or polyI:C induced significant apoptosis after 48h of stimulation (mean ±SE: 30.6 ±3% or 20 ±4% respectively), whereas no apoptosis was shown following TLR2 and TLR4 triggering by PGN and LPS. Pre-exposure of cells to DX2 was found to abolish apoptosis induced by CH11 but not by polyI:C treatment.
Conclusions: Cultured SGEC are susceptible to the induction of apoptotic cell death by polyI:C at comparable levels to the apoptosis induced by the anti-Fas mAb. Despite the induction of Fas expression, polyI: C-induced cell death appears Fas-independent.

080 DIFFERENTIAL EXPRESSION OF ACTIVATING AND INHIBITORY FC γ RECEPTORS IN HEALTHY AND RHEUMATIC SYNOVIA AND INHIBITION BY GLUCOCORTICOID

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Background: Autoantibody production is a characteristic of most autoimmune diseases including rheumatoid arthritis (RA). The formation of the autoantibodies with self-antigen into immune complexes makes them potent inducers of inflammation as they can activate Fc receptors on leukocytes. Fc gamma receptors (FcγRs) bind to the Fc part of IgG antibodies. Cross-linking of these receptors by IgG immune complexes lead to release of inflammatory cytokines.
Aim and Method: To investigate the expression of the different FcγRs; FcγRI, II and III, in synovial biopsies from joints of healthy individuals and RA patients, using immunohistochemistry and immunofluorescence. A recently developed antibody against the inhibitory FcγRIIB isoform was also used in the studies. We also wanted to examine if local treatment with glucocorticoid in RA joints had an effect on the expression of FcγRs.
Results: We found that FcγRII and FcγRIII were the FcγRs most expressed in RA synovial tissue, while FcγRI was least expressed. There was no difference in the expression of the different FcγRs at early stage of disease compared to a later stage of RA. Interestingly, a significant decrease in the expression of FcγRI and FcγRII was found in the RA synovia after glucocorticoid treatment, while no effect was seen on FcγRIII. In healthy synovia, FcγRI was not detected and the expression of FcγRII and III was significantly lower than in RA synovia. The inhibitory receptor FcγRIIB was not or very little expressed in healthy synovia compared to the high expression of FcγRIIB in RA synovia. All FcγRs were co-expressed with CD163, suggesting macrophages as the effector cells responsible for IgG-mediated inflammation in synovial tissue. Several of the FcγR-positive macrophages were accumulated around vessels and some FcγR positive cells were also detected inside vessels. Interestingly, the FcγR-positive macrophages were found adjacent to infiltrating T-cells also detected perivasculary.
Conclusion: All FcγRs are up regulated in RA synovia and glucocorticoid treatment of joints decrease the expression of FcγRI and FcγRII. The lack of FcγRI in healthy synovia and the reduction of FcγRI by glucocorticoid treatment, followed by suppressed joint inflammation, suggest that this receptor may be important for the ongoing inflammation seen in RA.

081 ROLE OF THE CHOLINERGIC NERVOUS SYSTEM IN COLLAGEN INDUCED ARTHRITIS IN MICE

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Objective: The nervous system, through the vagus nerve, can down-regulate inflammation in vivo by decreasing the release of tumour necrosis factor-α by stimulated macrophages. The vagus nerve exerts its anti-inflammatory effects by a specific effect of its principal neuro-transmitter acetylcholine on nicotinic acetylcholine receptors, in particular those of the α7 subtype, on macrophages. Most current strategies for treatment of rheumatoid arthritis (RA) are based on direct suppression of pro-inflammatory cytokines or cytokine activity. The identification of the cholinergic anti-inflammatory pathway now suggests several new approaches to modify cytokines and inflammatory responses to therapeutic advantage. The objective of our study is to obtain insight into the role of the cholinergic anti-inflammatory pathway in RA. We have investigated this in a well-known animal model for RA, namely collagen-induced arthritis (CIA) in mice.
Methods: We examined both the inhibition of the cholinergic anti-inflammatory pathway using vagotomy of the vagus nerve or stimulation of this pathway by administration of nicotine, which stimulates the α7 subunit of the nicotinic acetylcholine receptor. CIA was induced in DBA/1 mice at day 0 by bovine collagen type II followed by a booster injection on day 21. Unilateral cervical vagotomy or sham operation was performed on day -4 (8 mice per group). In a separate group of mice nicotine was added to the drinking water (50 μg/ml, 100 μg/ml and 150 μg/ml) from day -4 until sacrificing (8 mice per group). Disease progression was repeatedly monitored by visual clinical scoring and measurement of paw swelling with a caliper. Inflammation and joint destruction were assessed radiologically and histologically using a semiquantitative scoring system.
Results: Stimulation of the pathway by nicotine did reduce the arthritis activity in a reversed dose-dependent manner (for the 50 μg/ml treated group $p<0.05$ compared to control). In addition, we showed that vagotomy aggravate CIA compared to sham-operated mice ($p<0.05$). Radiographic analysis showed a trend towards protection against joint destruction in the knee and ankle joints of the mice treated with nicotine and an increase of damage in the vagotomy group compared to the sham-operated mice. Histological analysis revealed a reduction in synovial inflammation ($p<0.05$).
Conclusion: These data provide the first evidence that there is a role for the cholinergic anti-inflammatory pathway in the development of CIA in mice.

082 IS NIP3 THE CRITICAL MEDIATOR IN METHYLPREDNISOLONE INDUCED APOPTOSIS?

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Background: A lot of controversy exists about resistence to apoptosis of synovovial T lymphocytes, but it was recently demonstrated that SF T cells are susceptible to glucocorticosteroid-induced apoptosis. Although glucocorticosteroid induced apoptosis is a well known phenomenon, the underlying molecular mechanisms remain unknown.
Objective: To elucidate the molecular mechanisms of glucocorticoid-induced cell death.
Methods: Total RNA of purified T cells was prepared before, 6, 12 and 24h after treatment with 50 microM methylprednisolone (MP) in vitro, and analysed after multiplex ligation-dependent probe amplification (MLPA). Probes used in this study were designed to hybridise with cDNA of 36 apoptosis-related genes.

Apoptotic and necrotic cells were determined by microscopy and annexin V-PI staining. Intracellular localisation of Bax and cytochromeC were visualised using fluorescent microscopy. For detection of the mitochondrial transmembrane potential, cells were stained with JC-1 and oxidation of the dye DCF was used to measure reactive oxygen species (ROS) production. FACS was used for measurement of cytochrome C release and determination of caspase-3.
Results: After in vitro MP treatment, we found a specific upregulation of the mRNA of the pro-apoptotic Nip3 and its related protein Nip3L both in SF and PB T lymphocytes. No other significant changes were detected in any of the other 35 apoptisis related genes. The upregulation of the Nip3 expression was subsequently confirmed by Western blotting and fax staining. We next found that this leads to a aponecrotic cell death which is caspase-indepent, and primarily dependent on mitochondrial generated free radicals. We found that Bax translocated to the mitochondria concomitant with mitochondrial depolarisation and subsequent cytochrome C and ROS release. Addition of the pan-caspase inhibitor zVAD did not alter loss of mitochondrial membrane potential or cytochrome C release, indicative for caspase-independent initiation of the (intrinsic) mitochondrial apoptosis pathway. But, although zVAD inhibited caspase 3 activation, it failed to prevent cell death. Addition of rotenone, which inhibits mitochondrial respiratory chain complex I, abolished the measured ROS increase, demonstrating the mitochondrial origin of the free radicals. Whereas addition of zVAD failed to prevent cell death, addition of rotenone strongly inhibited cell death.

MP treatment in an oxygen deprived environment prevented apoptosis, and treatment with NAC, partly inhibited cell death, underscoring a central role for ROS in prednisolone induced cell death.
Conclusion: In vitro treatment of T lymphocytes with MP resulted in upregulation of the pro-apoptotic proteins Nip3. This proceeded an aponecrotic cell death, where mitochondrial produced free radicals play a central role in determining the cellular fate after corticosteroid treatment. This helps to explain the recently described susceptibility of synovial T cells to MP-induced apoptosis in RA. Additionally, insight into these mechanisms might help to enhance the therapeutic efficacy of corticosteroid treatment in RA patients.

083 INHIBITION OF CHRONIC DESTRUCTIVE ARTHRITIS USING A NATURALLY OCCURRING SPECIFIC TLR4 ANTAGONIST

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Background: Toll-like receptors (TLRs) belong to the family of pattern recognition receptors and are involved in the activation of both innate and adaptive immune responses by pathogens. Current evidence indicates that TLRs, in particular TLR4, can recognise several endogenous ligands, which are produced upon degeneration of extracellular matrix of cartilage and might be abundantly present in arthritic joints. Therefore, TLR4 may contribute to the autoimmune processes ongoing in rheumatoid arthritis (RA).
Objective: To investigate the involvement of TLR4 activation in the development of autoimmune destructive arthritis
Methods: Specificity of a naturally occurring TLR4 antagonist was characterised using mouse peritoneal macrophages, human peripheral blood mononuclear cells (PBMCs) and dendritic cells (DCs). Effects of the antagonist alone or in combination with various TLR ligands were determined on cytokine production and DC maturation. In vivo, collagen-induced arthritis (CIA) was treated using repeated injections of the TLR4 antagonist before or after the onset of arthritis. Clinical score of joint inflammation and histological characteristics of arthritis were evaluated after treatment.
Results: The TLR4 antagonist strongly inhibited the production of IL-1β, TNFα and IL-6 by mouse peritoneal macrophages and human PBMCs selectively upon stimulation with the TLR4 ligand LPS, not by TLR2 and TLR3 agonists. The antagonistic activity was also observed upon stimulation with endogenous TLR4 ligands such as extra domain A of fibronectin. A ratio of 10:1 TLR4 antagonist to LPS was sufficient to completely block the cytokine production. The antagonist did not by itself induce the production of anti-inflammatory cytokines or corticosteroids and had no effects on DC maturation in terms of CD83, CD86 and MHCII expression.

Treatment of CIA using the TLR4 antagonist significantly suppressed the clinical signs of arthritis in both early-phase and established arthritis. Histological examination of the knee joints after prophylactic treatment revealed that specific TLR4 inhibition significantly prevented proteoglycan depletion from the cartilage matrix. Furthermore, destruction of the cartilage matrix and chondrocyte death was markedly reduced. In therapeutic setting, treatment with TLR4 antagonist resulted in reduction of proteoglycan depletion and destruction of cartilage matrix. Infiltration of inflammatory cells into the joint space and chondrocyte death was also strongly inhibited. In addition, another characteristic hallmark of CIA, bone erosion, was almost completely blocked. Importantly, the levels of antibodies against type II collagen (CII) were comparable between the treated and the control group.
Conclusion: Inhibition of TLR4 pathway substantially suppressed both clinical and histological characteristics of CIA. These protective effects solely relied on blocking of TLR4 signalling, as the antagonist did not disturb the development of the adaptive anti-CII immune response. Our data provide evidence for the involvement of TLR4 signalling in the pathogenesis of RA and suggest that TLR4 may be a novel target in the treatment of RA.

084 PI 3-KINASE DEPENDENT INACTIVATION OF THE FOXO4 TRANSCRIPTION FACTOR IN RHEUMATOID ARTHRITIS SYNOVIAL MACROPHAGES

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Objectives: PI 3-kinase dependent activation of the proto-oncogene product Protein Kinase B (PKB) has been observed in rheumatoid arthritis (RA). Disruption of this signalling pathway ameliorates disease in animal models of arthritis. Recent evidence has shown that PKB-dependent phosphorylation and inactivation of FoxO family transcription factors is largely responsible for the anti-apoptotic effects of the PI 3-kinase signalling pathway. The purpose of this study was to determine if PKB-dependent inactivation of FoxO transcription factors is observed in RA synovial tissue, and if this signalling pathway is associated with clinical disease parameters.
Methods: Arthroscopic synovial biopsies were obtained from 12 RA and 9 inflammatory osteoarthritis (OA) patients. Expression and PKB-dependent phosphorylation of FoxO1, FoxO3a, and FoxO4 transcription factors were determined using specific antibodies for immunohistochemical analysis. Double-labelling was performed to identify FoxO inactivation in specific populations.
Results: Each FoxO family member was expressed and phosphorylated in both RA and OA. FoxO1 was phosphorylated in RA fibroblast-like synoviocytes (FLS) and macrophages. Phosphorylated FoxO3a and FoxO4 were detected in synovial T lymphocytes and macrophages, respectively. No differences in FoxO family member expression were detected between RA and OA synovial tissue. However, specific phosphorylation of FoxO4, but not FoxO1 or FoxO3a, was significantly enhanced ($p<0.05$) in RA synovial sublining (18.9 arbitrary units, SEM 7.8) compared to OA tissue (11.9, SEM 7.4). In vitro, both TNF-α and CD40 stimulation resulted in FoxO4 phosphorylation in healthy donor blood-derived macrophages. Phosphorylation of FoxO4 in the RA intimal lining layer and synovial sublining layer negatively correlated with RA patient serum CRP levels (lining $R=-0.82$, $p=0.049$; sublining $R=-0.41$, $p=0.024$) and ESR (lining $R=-0.74$, $p=0.034$; sublining $R=-0.77$, $p=0.034$).
Conclusion: Our studies demonstrate that PI3-kinase dependent phosphorylation of the FoxO4 transcription factor in synovial sublining macrophages distinguishes RA from OA. Inflammatory cytokine and cell-cell interactions, such as TNF-alpha and CD40 stimulation may contribute to inactivation of FoxO4 in RA synovial macrophages, protecting these cells from apoptosis.

085 EXPRESSION AND ACTIVATION OF P38MAPK ISOFORMS IN RHEUMATOID ARTHRITIS

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Activation of p38MAPK is a key signaling step of proinflammatory cytokines. Inhibition of p38MAPK has proven as an effective tool to inhibit experimental inflammatory disease and clinical studies testing inhibitors of p38MAPK are currently performed. It is unclear however, which specific molecule, among four different isoforms of p38MAPK (termed alpha to delta) is predominantly involved in the pathology of chronic inflammatory diseases, such as rheumatoid arthritis (RA). To test this we examined synovial tissue of patients with rheumatoid arthritis for the expression and activation of p38MAPK isoforms a, b, g and d by means of immunoblotting, immunoprecipitation and immunohistochemistry. Immunoblot analysis revealed p38MAPK a and g as predominantly expressed in synovial tissue of RA, whereas the two other isoforms were less frequently present. Immunohistochemistry revealed expression of p38MAPK isoforms in the synovial lining as well as blood vessels. Co-labeling with cell specific markers revealed that macrophages express the a- and g- isoforms, synovial fibroblasts the b- and g- isoforms, granulocytes the d-isoform whereas T- lymphocytes are rarely positive for any p38MAPK isoform. Double labeling with isoform-specific antibody and pan-p38 antibody against the phosphorylated form of p38MAPK showed activation of the a and g isoform in the synovial lining and endothelial cells. Occasional activation of the b-isoform was also denoted in the synovial lining and the endothelium, whereas the d-isoform, although expressed in pericytes around blood vessels, was not phosphorylated. This phosphorylation pattern was confirmed by immunoprecipitation of activated p38MAPK from synovial tissue extracts and identfying it as p38MAPKa and g but not p38MAPKb and d. These data show that the a and g - isoforms of p38MAPK are predominantly involved in synovial inflammation of RA and suggest that strategies to inhibit p38MAPK in inflammatory arthritis should aim to specifically target the a and g - isoforms of p38MAPK.

086 PDGF-BB INDUCES TGF-β PATHWAY GENE EXPRESSION IN SYNOVIAL FIBROBLASTS: STRONGER EFFECTS IN RHEUMATOID ARTHRITIS THAN IN OSTEOARTHRITIS

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Differential gene expression was investigated in early-passage rheumatoid arthritis (RA)- and osteoarthritis (OA)- synovial fibroblasts (SFB; n =6 each) before/after stimulation with PDGF-BB using Affymetrix arrays; mRNA/protein data were validated by real-time RT-PCR and/or western blots.

Using Affymetrix arrays, RA-SFB showed constitutive upregulation of components of the TGF-β/BMP-pathway TGF-β1, its receptor TbetaRI, the TGF-β binding proteins LTBP1/2, the TGF-β-releasing thrombospondin 1 (Tsp1), and the smad-associated molecule SARA (fold-change between 1.2 and 3.4), but downregulation of BMP-7 and BMP-4 (fold-change of 0.5 and 0.4). Constitutive upregulation of TGF-β 1 and Tsp1 in RA-SFB was confirmed by real-time PCR; in addition, RA-SFB showed a constitutively lower expression of c-jun.

Following PDGF-stimulation (2 h; Affymetrix), RA-SFB showed significantly weaker induction of c-fos and c-jun than OA-SFB (fold-change of 0.39 and 0.63), and strikingly TGF-β/BMP-pathway related genes, i.e., the BMP-receptor-triggered smad5 (0.68-fold), the TGF-β signal inhibiting snoN (0.17-fold), and the TGF-β-induced PAI-1 (0.5-fold), but stronger induction of TGF-β-induced uPA (1.46-fold). Only marginal differences were observed at 24 h.

Real-time PCR showed significant upregulation of most mRNA species by PDGF-BB (2 h), either selectively in RA-SFB (c-jun, snoN) or OA-SFB (PAI-1) or in both RA-and OA-SFB (TGF-β1, Tsp1, TβRI, smad5, and uPA). Significant differences between stimulated OA- and RA-SFB were observed for c-fos (RA<OA), Tsp1, and TβRI (both RA>OA). At the protein level, increased expression following stimulation with PDGF-BB was observed in both RA- and OA-SFB for c-fos (4 and 8 h; $p\leqslant0.05$ only for OA-SFB), TGF-β1 (8 and 16 h; RA>OA; $p\leqslant0.05$), TβRI (16 h), and snoN (4, 8, and 16 h). RA-SFB show broad alterations of the TGF-β-pathway, both constitutively and following PDGF-BB stimulation. Augmented production of TGF-β mRNA/protein by RA-SFB, in conjunction with the presence of TβRI, suggests a pathogenetic role of TGF-β-induced effects on.

087 ACTIVATION OF THE INTERFERON-STAT1 PATHWAY IN RHEUMATOID ARTHRITIS PERPHERIAL MONOCYTES AND SLE LYMPHOCYTES AND MONOCYTES

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Aim: Type I interferons, e.g. IFN α, and type II interferon (IFN gamma) signal via activation of Stat1 (Signal transducer and activator of transcription 1). Both type of IFNs have pathogenic relevance in SLE, and increased expression and activation of Stat1 was shown in synovial tissue from RA (rheumatoid arthritis) patients. We therefore examined the expression and phosphorylation of Stat1 in SLE and RA PBMC.
Methods: PBMC were isolated over Ficoll-Hypaque gradients, and intracellular immunofluorescence staining for Stat1 and pStat1 was performed immediately after preparation or after 15 minutes of incubation in medium with or without the addition of 100 U/ml IFN α or 100 U/ml IFN γ. Cells were analysed on a FACScan fluorocytometer immediately after staining and gates were set for monocytes and lymphocytes.
Results: The IFN-inducible Stat1 protein was increased in SLE PBMC as compared to healthy controls (HC) and RA cells (Lymphocytes: SLE: 16.2 ± 13.2 (mean $\pm$ SD); HC: 5.3 ± 1.9; RA: 4.5 ± 3.0; Monocytes: SLE: 17.8 ± 12.6; HC: 7.4 ± 3.3; RA: 6.8 ± 5.6;). Both SLE lymphocyte and SLE monocyte Stat1 mfi positively correlated with SIS ($r=0.65$, $p<0.0001$ and $r=0.48$, $p<0.0005$, respectively), and with parameters associated with SLE activity, such as anti-dsDNA. No association was found between RA PBMC Stat1 levels and the DAS28.

Baseline phospho-Stat1 (pStat1) was increased in SLE versus healthy and RA lymphocytes (SLE: 1.64 ± 0.36; HC: 1.37 ± 0.2; RA: 1.41 ± 0.2). Interestingly, however, both SLE and RA monocytes contained more activated Stat1 (pStat1) than HC monocytes (SLE: 4.53 ± 1.79; RA: 5.17 ± 1.31; HC: 3.35 ± 0.92).

Incubation with IFN α similarly increased Stat1 phosphorylation in SLE, RA and healthy lymphocytes as compared to incubation in medium alone. In contrast, IFN γ significantly induced Stat1 phosphorylation in SLE lymphocytes (from 1.56±0.25 to 1.72±0.37, p<0.002), and not in those of RA patients (from 1.39±0.23 to 1.44±0.32, p=NS) and healthy individuals (from 1.39±0.16 to 1.39±0.17, p=NS). However, the increase in pStat1 upon stimulation with IFN gamma was much more pronounced in SLE (from 4.1±1.2 to 6.9±3.3, p<0.0001) and RA monocytes (from 4.8±1.6 to 6.8±2.9, p<0.005) than in healthy monocytes (from 3.5±0.9 to 4.4±1.5, p<0.005).
Conclusions: Activity-associated Stat1 overexpression is found in SLE but not RA, possibly due to increased interferons. Hyperphosphorylation of Stat1 is found in SLE lymphocytes as well as in RA and SLE monocytes, all of which appear primed to overreact to IFN γ.

088 MODULATION OF GENE EXPRESSION PROFILES OF PBMC IN RHEUMATOID ARTHRITIS PATIENTS TREATED WITH ANTI-CD20 (RITUXIMAB)

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Although the precise role of B cells in the pathogenesis of rheumatoid arthritis is not yet clear, these cells are like other cell populations involved in the inflammatory process. A new approach in the treatment of rheumatoid arthritis are B cell depletive therapies which have been proven to exert beneficial effects in RA patients. Rituximab is a monoclonal antibody directed against CD20, an antigen expressed by B cells but not B-cell progenitor or plasma cells. Using the microarray technique, changes in the gene expression pattern following an anti-CD20 therapy of rheumatoid arthritis (RA) patients were analysed.

The study included 5 RA patients who had failed to respond to classical DMARDs and TNFα blocking therapy. Before the start of treatment, all patients were characterised by a high disease activity (DAS28 mean 6.4; range 4.6–7.2). Patients received 500 mg rituximab weekly for four weeks.

Blood samples were taken before, 3 and 6 days after initiation of therapy. Total RNA from mononuclear cells was prepared with the RNeasy kit (Qiagen, Germany). Affymetrix chip technology was used to analyse the expression levels of about 22,000 transcripts (Human genome chip U133A). To confirm Affymetrix chip data RNA expression levels of selected genes were compared by quantitative real-time PCR.

Within 3 months after the start of treatment a clinical improvement was seen in all patients, as reflected by a reduction of DAS28 by 37.3% (mean range between 22.9% and 66.5%). Significant decreases in expression levels at day 3 and/or 6 compared to baseline were seen in all patients in genes which encode B cell proteins such as CD19, CD20, CD22, the B cell linker protein and others. For some of these genes no specific RNA was detectable on the microarray only 3 or 6 days after treatment, suggesting an effective depletion of B cells. A similar result was seen in those genes for immunoglobulins (different classes, heavy and light chains) reaching signal log ratios between −1.28 and −3.31 (mean).

Interestingly, most of the genes that were found differentially expressed in RA patients in response to TNFα blocking therapy were almost unaffected by anti-CD20 treatment. Expression levels of the key inflammatory cytokines TNFα and IL-1β remained unchanged as well as levels of COX-2, PDE4B, IL-8, ICAM and others as confirmed using TaqMan RT-PCR analysis.

Our results show that the therapy with rituximab results in numerous transcriptional changes which are due to the disappearance of B cells.

In contrast to TNFα blocking therapy, the therapy response due to B cell depletion seen in our patients was not associated with early changes in gene expression downstream or upstream to TNFα or IL1β, suggesting the existence of other mechanisms responsible for disease activity and therapy outcome.

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089 A NOVEL ROLE FOR THE HDL RECEPTOR CLA-1 IN SYNOVIAL INFLAMMATION THROUGH SERUM AMYLOID-A AND APOPROTEIN-A1

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Introduction: The acute phase apoprotein Serum Amyloid A (A-SAA) is elevated in serum (1–1000 μg/ml) and correlates with disease activity in rheumatoid arthritis (RA). Our group has previously demonstrated A-SAA induction of angiogenesis, adhesion molecule expression and MMP production from RA synovial fibroblast cells (RASFC) and microvascular endothelial cells (EC) in vitro, and marked expression of A-SAA and apolipoprotein A1 (Apo-A1) in RA synovial membrane (SM). A-SAA is known to signal through a G-coupled receptor FRPL-1, but recent evidence suggests an alternative receptor pathway, the novel high density lipoprotein (HDL) receptor CLA-1 (CD36 and LIMPII analogous-1) may be involved.
Aims: To determine expression and localisation of CLA-1 in human synovial tissue, RASFC and EC and to demonstrate a functional role for CLA-1 in A-SAA induced inflammatory responses in RA.
Methods: Quantitative expression and localisation of CLA-1 in RA SM, RASFC lines (n=5) and in EC was assessed by Western blotting analysis and immunohistology/fluorescence. Cell surface expression of inter-cellular adhesion molecule-1 (ICAM-1) was assessed by flow cytometry. The effects Apo-A1 (10μg/ml) and the inhibitor of FPRL-1, lipoxin-A4 (LXA4) (500 ng/ml) on A-SAA mediated ICAM-1 expression in SFC was assessed by flow cytometry. Statistical analysis was performed using SPSS 11 software.
Results: Synovial expression of CLA-1 was localised to the lining layer and endothelial/perivascular regions, consistent with the distrubution of A-SAA and Apo-A1 in SM. Western blot analysis and immunofluorescence demonstrated protein CLA-1 in EC and in all 5 RASFC lines. ICAM-1 was significantly upregulated from basal median flourescence intensities (MFI) of 42–63MFI to 78-139MFI (p<0.05) following A-SAA stimulation and 85–112MFI (p<0.05) following TNFα stimulation. A-SAA induced ICAM expression correlated significantly with CLA-1 expression in RASFC (p<0.05). There was no correlation between TNFα induced ICAM expression and CLA-1 expression. A dose dependent inhibition of A-SAA induced ICAM-1 expression was seen with both Apo-A1 and LXA4. A-SAA (1μg/ml) upregulation of ICAM-1 was reduced by 73% in the presence of Apo-A1 and 60% in the presence of LXA4 (p<0.05, n=3).
Conclusions: In addition to the FRPL-1 receptor, these data suggest that the novel HDL receptor CLA-1 may act as an alternative pathway for the A-SAA induced pro-inflammatory response locally in the joint. Opposing actions of A-SAA and Apo-A1 on RA SFC responses in vitro suggest that they may modulate synovial inflammation in vivo through the CLA-1 receptor.

090 INTERFERONS LIMIT CELL CYCLE PROGRESSION IN FIBROBLAST-LIKE SYNOVIOCYTES AND INDUCE EXPRESSION OF P21WAF1/CIP1

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Introduction: Accumulating evidence suggests that fibroblast-like synoviocytes (FLS) play crucial roles in both joint damage and the propagation of inflammation. Recently, we provided evidence for activation of STAT-1 in RA synovial tissue, which was particularly prominent in FLS.
Aim: To gain more insight into the functional effects of STAT-1 activation in RA FLS and its relevance for RA pathology, we have examined the effect of IFN-induced STAT-1 activation on FLS survival and growth.
Methods: Immunocytochemistry was performed to analyse STAT-phosphorylation and nuclear translocation. Proliferation was evaluated by measuring DNA synthesis through the incorporation of [3H]-Thymidine. For cell cycle analysis and identification of apoptosis, cells were stained with propidium iodide (PI) or Annexin V-FITC and PI, respectively, and analysed by flow cytometry. Real-time PCR analysis was applied to measure the expression of target genes.
Results: Treatment of RA FLS with IFNα, IFNβ or IFNγ led to rapid phosphorylation of STAT-1 and induction of a STAT-1-specific transcriptional response, indicating that the IFN response was functional in RA FLS. Measurement of the proliferation rate revealed that IFNα, IFNβ and IFNγ suppressed RA FLS proliferation and that this effect overruled the stimulatory effect of TNFα. No significant difference in the levels of apoptosis in RA FLS treated with IFNα, IFNβ or IFNγ was observed compared to unstimulated cells, suggesting that the inhibitory effect was not due to induction of apoptosis. Analysis of the cell cycle distribution revealed that whereas TNFα treatment induced cell cycle progression to G2/M phase, treatment with IFNα, IFNβ or IFNγ limited cell cycle progression and caused accumulation of cells in G0/1 phase. Accordingly, we found that treatment of RA FLS with IFNα, IFNβ and IFNγ induced expression of the cyclin dependent kinase inhibitor (CDKI) p21waf1/Cip1, which inhibits CDK activity and may therefore mediate the IFN-induced accumulation of RA FLS in G0/1 phase.

Conclusion: IFN treatment suppressed RA FLS proliferation by limiting cell cycle progression, which correlated with induction of the CKDI p21waf1/Cip1. These results indicate that IFN-treatment can negatively modulate the growth abnormalities of FLS, suggesting that therapeutic approaches that selectively manipulate STAT-1 may have beneficial effects in RA.

091 TLR9 AGONISTIC CPG-OLIGODEOXYNUCLEOTIDES AND IL-15 STIMULATE ACTIVATION, PROLIFERATION, AND DIFFERENTIATION OF BONE MARROW B LYMPHOCYTES FROM RHEUMATOID ARTHRITIS PATIENTS

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Background: Bone marrow (BM) creates a specific microenvironment, rich in survival and growth factors, that promote the development and maintenance of humoral immunity. Recently it was also shown, that effective antigen presentation and T cell activation takes place in bone marrow. In our previous studies we could show that bone marrow B lymphocytes were activated by potent toll-like receptor 9 (TLR9) agonist, CpG oligodeoxynucleotides (CpG-ODN) in bone marrow mononuclear cells (BMMC) culture. These effects were blocked by inhibitor of TLR9 signaling, chloroquine. We also detected bacterial DNA the natural agonists of TLR9 in several RA bone marrow samples. In addition, the level of IL-15 protein, known B cell activating factor, was significantly increased in bone marrow of RA compared to OA patients.
Aim: The aim of this study was to investigate the direct effects of TLR9 agonists (CpG-ODN) and IL-15 stimulation on purified RA bone marrow B lymphocytes.
Material and Methods: BMMC were isolated from RA and OA patients during hip replacement surgery. CD20 positive cell mature B cells were sorted from RA BMMC using MACS technique. B cells were cultured in vitro in a presence of agonistic CpG-ODN or control GpC-ODN alone or with addition of IL-15. The expression of TLR9, activation markers (CD86, CD69, CD54) and proliferation marker Ki-67 on B lymphocytes were analysed using flow cytometry. B cell subpopulations were analysed according to CD19, CD20, CD27 and CD138 surface expression.
Results: CpG-ODN, but not GpC-ODN stimulation triggered enhanced expression of CD69, CD86, CD54, and Ki-67 on sorted CD20+ BM B cells. CpG-ODN and IL-15 exerted additive effects on CD69, CD86 and TLR9 expression. IL-15 was more potent than CpG-ODN in triggering B cell proliferation. However, only CpG-ODN induced differentiation of sorted B cells into CD19+CD20+CD27high cells. Simultaneous treatment of B cells with CpG-ODN and IL-15 promotes further differentiation of CD19+CD20+CD27high cells, accompanied by up-regulation of CD138 and down-regulation of CD19 and CD20 expression, suggesting their transition towards plasma cells. Interestingly, we found higher number of CD19+CD20medCD27highCD138+ cells in BM obtained from RA than OA patients. Increased number of CD86+ B cells in RA vs OA BM samples was also observed.
Conclusions: Our results indicate that TLR9 agonistic CpG-ODN directly trigger BM-derived B-cells from RA patients to: (1) up-regulate costimulatory and adhesion molecules (CD69, CD86, CD54) expression; (2) enhance cell proliferation, and (3) differentiate into CD19+CD20+CD27high cell population. IL-15 and GpG-ODN exert additive effects on CD69, CD86, TLR9 expression and proliferation of B cells. IL-15 participates also in CpG-ODN-induced B cell differentiation.

Our results suggest that activation of bone marrow B cells by present in situ TLR9 ligands and/or IL-15 may contribute to the pathogenesis of RA.

092 TUMOUR NECROSIS FACTOR ALPHA (TNFα) ATTENUATES IL-2 GENE EXPRESSION BY TARGETTING TRANSCRIPTIONAL ACTIVITY OF SPECIFIC NF-κB AND AP-1 SUBUNITS

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Background: IL-2 is essential for the development of peripheral T cell tolerance, since IL-2-deficient mice develop lymphoproliferative disease and lethal autoimmunity. This pathology may be explained in part by the dependence of regulatory T cells (T regs) on IL-2 for expansion and suppressor function. T regs do not make IL-2, local effector T cells being the probable source of this cytokine. Thus failure of effector T cells to produce IL-2 may allow development of autoimmunity. Indeed, suppressed transcription of IL-2 is a feature of many autoimmune diseases, including rheumatoid arthritis (RA). RA synovial T cells, despite having an activated, memory phenotype, do not express IL-2 when stimulated ex vivo. Notably, this hyporesponsiveness is reversed following anti-TNF therapy. These events can be modelled in vitro: we have shown that culture of mouse T cell hybridoma 11A2 in TNF suppressed subsequent induction of IL-2 mRNA and protein by up to 95%. TNF had little effect on stability of IL-2 message, therefore we examined effects of TNF pre-treatment on transcriptional activation of the IL-2 gene. We have focussed on transcription factors NF-κB, NFAT and AP-1, which contribute to IL-2 gene activation via inducible binding at the proximal promoter (pIL-2).
Aim: To determine the effects of TNF on transcriptional activation of the IL-2 gene in T cells.
Methods: 11A2 cells were cultured with or without TNF (2.5 ng/ml) for 8 days. Control and TNF-treated cells were transfected with plasmid expressing luciferase under the control of specific transcription factor-binding elements. Transcriptional activities of NF-κB, NFAT/AP-1 or AP-1 were then measured by luciferase expression, following stimulation with phorbol ester and ionomycin. Nuclear translocation and DNA consensus binding of these transcription factors were assayed by immunoblot and electrophoretic mobility shift assay (EMSA) of stimulated cell fractions, respectively.
Results: In TNF-treated T cells, transcriptional activation of consensus and variant (CD28 response region of pIL-2) NF-κB elements was suppressed by 80%, and activation of both a simple AP-1 element and the composite NFAT/AP-1 element of pIL-2 by 55%, as compared to controls. Nuclear translocation of c-Rel, p65, c-fos, and fosB, but not NFAT, c-jun or junB, was markedly reduced in TNF-treated cells relative to controls. EMSA showed attenuation of induced binding of 11A2 nuclear proteins to an NF-κB oligonucleotide following TNF-pre-treatment, as well as some reduction in induced AP-1 factor binding.
Conclusion: We demonstrate that TNF pre-treatment inhibits the nuclear expression, DNA binding, and transcriptional activities of NF-κB and fos family members. This may contribute to TNF-mediated blockade of IL-2 induction in T cells. Our data define a crucial link between effects of inflammation and impaired peripheral T cell self-tolerance.

093 CORTICOTROPIN RELEASING FACTOR AND THE UROCORTINS AFFECT MACROPHAGE ACTIVATION VIA DISTINCT SIGNALING PATHWAYS

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Corticotropin releasing factor (CRF) and its related peptides Urocortin 1 (UCN1) and Urocortin 2 (UCN2) are paracrine regulators of inflammation. CRF and UCN1 are expressed in the synovium of patients with rheumatoid arthritis. Inhibition of CRF signals attenuates inflammation in several experimental models. We have previously shown that CRF augments LPS-induced pro-inflammatory cytokine secretion from macrophages. Aim of the present study was to determine the mechanism by which CRF and its related peptides Urocortin 1 (UCN1) and Urocortin 2 (UCN2) affect macrophage sensitivity to LPS. Exposure of primary mouse peritoneal macrophages to CRF, UCN1 or UCN2 resulted in increased expression of the LPS receptor TLR4, as documented by an increase of its protein and transcript levels. The effect of CRF peptides on TLR4 gene transcription was confirmed in RAW264.7 cells transfected with a minimal TLR4 promoter linked to the luciferase gene. CRF peptides induced nuclear translocation and DNA binding of the transcription factors PU.1 and AP1, key elements for the regulation of the TLR4 promoter. The effects of CRF peptides were inhibited by the CRFR2 antagonist anti-sauvagine30 but not by the CRFR1 antagonist antalarmin. Moreover, CRF peptides prevented the inhibitory effect of LPS on TLR4 expression, suggesting that they may negatively affect macrophage tolerance to LPS.

The effect of CRF peptides on pro-inflammatory cytokine secretion appears to be biphasic. Thus, treatment of primary mouse macrophages with CRF, UCN1 or UCN2 results in an acute suppression of LPS-induced TNF-α secretion. This effect occurred via induction of Cox-2 and secretion of PGE2. CRF- and UCN-induced PGE2 secretion was mediated by the PI3K/Akt1 pathway. Homologous deletion of CRFR1 and CRFR2 in mice confirmed our observations indicating that endogenous CRF peptides contribute in the regulation of macrophage sensitivity to pro-inflammatory stimuli.

In conclusion, our data suggest CRF and the urocortins exert a biphasic paracrine effect on macrophage activation. In the early phase of inflammation, CRF and the urocortins act as anti-inflammatory agents suppressing TNF-α release via induction of Cox-2 expression and PGE2 production. However, at a later stage, CRF and the urocortins augment

LPS-induced pro-inflammatory cytokine production via upregulation of TLR4, thus acting as pro-inflammatory agents.

094 T CELLS EXPRESSING LOW LEVELS OF TCRZETA PREFERENTIALLY MIGRATE TO SITES OF INFLAMMATION

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Background: The zeta chain of the T cell receptor (TCR) complex is vital for signal transduction and TCR/CD3 assembly. It is down-regulated in chronic inflammatory diseases such as SLE and rheumatoid arthritis (RA), leading to hyporesponsiveness to antigen stimulation. We have developed a FACS-based TCRzeta expression index and have identified a population of effector T cells in peripheral blood (PB) expressing low levels of TCRzeta (ie TCRzeta dim cells). These cells express markers of previous antigen exposure, retain the capacity to produce inflammatory cytokines and stimulate monocytes to produce TNFα in a cell contact-dependent manner. This effector phenotype closely resembles RA synovial T cells.
Methods: We explored whether TCRzeta dim T cells tend to migrate to inflammatory sites such as the inflamed synovial joint. Initially, we compared the transmigratory potential of TCRzeta bright or dim cells across monolayers of TNFα-stimulated human umbilical vein endothelial cells using transwell inserts. Fresh PB lymphocytes from healthy donors were added to the upper chamber of the system prior to sampling cells in the upper and lower chambers 24 hours later. Cells were stained with intracellular PE-conjugated anti-human TCRzeta antibodies after fixing/permeabilisation, together with cell surface markers (CD3, CD4, CD8 and CD45RA/RO), prior to FACS analysis. We also compared TCRzeta expression in paired PB and synovial fluid (SF) T cells from 5 patients with RA experiencing a disease flare, as well as 8 RA synovial samples. Finally, we studied PB TCRzeta dim cell subsets before and after infliximab therapy.
Results: After 24 hours, the TCRzeta bright/dim ratio within CD3, CD4 and CD8 subsets was reduced in the lower chamber, indicating that a greater proportion of TCRzeta dim cells had migrated. This was most noticeable for CD4+ T cells with a significant enrichment for CD45RO+ T cells in the migrating population. Further analysis confirmed that whilst 39% of migrating CD4+ T cells were TCRzeta bright, nearly 80% were TCRzeta dim. In the RA patients, levels of TCRzeta dim cells were highly variable, but surprisingly low levels were detectable in the PB of those with active disease. In contrast, significant expansion of the TCRzeta dim subset was observed in the SF and tissue compartment from the same patient, consistent with migration to inflamed joints and depletion from the PB. Finally, a good response to infliximab therapy was associated with accumulation of TCRzeta dim cells in the PB.
Conclusions: TCRzeta dim T cells migrate across endothelial barriers in vitro, and their numbers are increased in synovial inflammatory exudates relative to PB during RA flares, suggesting they are effector memory T cells with a predisposition to migrate to inflammatory sites. Exclusion of this T cell subset from inflamed tissues is associated with a good clinical response to TNFα blockade.

Evolving concepts in synovitis: cytokine, chemokines, and fibroblasts

095 EXPRESSION OF INTERLEUKIN-22 IN RHEUMATOID ARTHRITIS: POTENTIAL ROLE AS A PROINFLAMMATORY CYTOKINE

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Objective: IL-22 is a novel IL-10 family cytokine. While its pathophysiological function is largely unknown, induction of acute phase responses by IL-22 has suggested proinflammatory properties. In this study, we sought to examine whether IL-22 plays a role in the pathogenesis of rheumatoid arthritis (RA).
Methods: Expression of IL-22 and IL-22 receptor 1 (IL-22R1) was examined by reverse transcriptase-PCR (RT-PCR), Western blot, and immunohistochemical analysis. The effects of recombinant IL-22 (rIL-22) on cultured synovial fibroblasts derived from RA patients (RASF) for proliferation and monocyte chemoattractant protein-1 (MCP-1) production were examined by alamer Blue assay and ELISA, respectively.
Results: IL-22 mRNA was detected by RT-PCR in RA synovial tissues and mononuclear cells isolated from RA synovial fluids. High levels of IL-22 were expressed both in the lining and sublining layers of RA synovial tissues. Staining for vimentin and CD68, as markers of synovial fibroblasts or macrophages, respectively, showed that the majority of IL-22-positive cells were synovial fibroblasts and macrophages. IL-22R1 was also expressed in both the lining and sublining layers of RA synovial tissues. The majority of cells expressing IL-22R1 were positive for vimentin, but not for CD68. Expression of IL-22 and IL-22R1 in RASF was confirmed by RT-PCR and Western blot analysis. rIL-22 at 100 ng/ml induced approximately a four fold increase in proliferation and a five fold increase in MCP-1 production by RASF above medium controls.
Conclusions: These data suggest that IL-22, produced by synovial fibroblasts and macrophages, promotes inflammatory responses in RA synovial tissues by inducing the proliferation and chemokine production of synovial fibroblasts.

096 RHEUMATOID ARTHRITIS SYNOVIUM CONTAINS IMMATURE MYELOID AND PLASMACYTOID DENDRITIC CELLS WITH DISTINCT CYTOKINE PROFILES

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Background: Synovial tissue (ST) of rheumatoid arthritis (RA) patients is characterised by synovial lining hyperplasia and chronic infiltration by T and B cells, monocyte/macrophages, dendritic cells (DC) and other cells. DC have been proposed to play a role in the initiation and perpetuation of RA by presentation of arthritogenic antigen to autoreactive T cells. Moreover, cytokines play a crucial role in the pathogenesis of RA. In this respect, the RA ST is associated with an increased production of an array of cytokines by several cell types. We undertook this study to gain insight into the in vivo characteristics of two major DC subsets, myeloid (m) and plasmacytoid (p)DC, in RA ST, by investigating their frequency, phenotype, distribution and cytokine expression.
Methods: ST was obtained from 24 patients with RA, 10 with psoriatic arthritis (PsA) and 12 with inflammatory osteoarthritis (OA). Immunohistochemistry of ST was performed using specific antibodies against mDC and pDC and stained sections were evaluated by digital image analysis. Coexpression and distribution of mDC and pDC with lineage markers and T cell markers was performed by double immunolabelling. To evaluate the maturation status and the coexpression of cytokines by mDC and pDC, double immunofluorescence microscopy was used and the numbers of double positive DC were quantified.
Results: In all diagnostic groups studied the number of pDC was significantly higher compared with mDC. In RA ST both mDC and pDC were colocalised to T cell aggregates. Here we show for the first time that mDC and pDC produce locally distinct cytokine profiles. The percentage of pDC expressing IL-18 and IFN-a was significantly higher, and the percentage of pDC expressing IL-12p70 and IL-23p19 was significantly lower compared with mDC.
Conclusion: Our results indicate that mDC and pDC in ST may play an important role in synovial inflammation possibly via stimulation of memory T cells. Moreover, the release of proinflammatory and Th1-inducing cytokines by ST DC may sustain the inflammatory process. In this respect, IL-12 together with IL-18 may induce/enhance IFN-γ production by effector T cells, type I IFNs may play a role in (auto)antibody production by B cells and IL-23 the induction of the newly described Th17 T cell subset. Conceivably, immunomodulation by targeting ST DC might provide a novel antirheumatic strategy.

097 THE ROLE OF TNF AND IL-17 IN THE PROGRESSION OF IL-1 DRIVEN EXPERIMENTAL ARTHRITIS

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Background: IL-1 receptor antagonist-deficient (IL-1Ra−/−) mice spontaneously develop an inflammatory and destructive T cell-driven arthritis due to unopposed excess IL-1 signaling. Previous studies have shown that the development of IL-1Ra-/- arthritis is markedly suppressed by TNF-deficiency, and is completely blocked in the absence of IL-17. Although these studies demonstrated the importance of TNF and IL-17 in the development of the IL-1Ra−/− arthritis model, the role of these

cytokines in the progression of this arthritis model remains to be elucidated.
Objective: To study the role of TNF and IL-17 in the progression of the spontaneous IL-1Ra−/− arthritis model by neutralising these cytokines after the onset of arthritis.
Methods: Development of spontaneous arthritis was determined by macroscopic scoring on a scale of 0–2 per paw. Arthritic animals with a clinical score of 0.75–1.0 were treated with intraperitoneal injections of sTNFRI(p55) (10 mg/kg), anti-IL-17 serum (0.2 ml) or control rabbit serum (0.2 ml) (at day 0 and 4). Treatment with anti-IL-1 was also included in this study. Severity of arthritis was scored macroscopically, and ankle joints were isolated for histological analysis at day 7 after start of treatment.
Results: Previously was shown that IL-1Ra−/− T cells produce markedly enhanced IL-17 levels upon stimulation with anti-CD3, and that T cell transfer could induce arthritis in recipient mice. In this study was shown that blocking of IL-17 after onset of IL-1Ra−/− arthritis clearly halted progression of the macroscopic arthritis scores, and significantly suppressed joint inflammation and bone erosion compared to the control-treated mice. In contrast, treatment with sTNFRI(p55) did not prevent aggravation of both clinical and histological scores. As expected, anti-IL-1 was able to reduce the disease. These data indicate that IL-17, in contrast to TNF, is involved in the progression of inflammation and destruction in this spontaneous T cell-driven arthritis model.

Subsequently, the role of TNF in IL-1-induced joint pathology was further investigated using adenoviral IL-1 overexpression in TNF-deficient mice. Despite high local IL-1 levels in patella-washouts (810 pg/ml) at day 1 after virus injection (10.7 pfu i.a.), hardly any TNF production could be detected (4,7 pg/ml). In line with this, IL-1-mediated joint pathology was not suppressed in TNF-deficient mice, indicating that IL-1-induced joint inflammation and destruction are independent of TNF.
Discussion: Our blocking study in IL-1 receptor antagonist-deficient mice shows that even after onset of arthritis, IL-17 is involved in joint inflammation and destruction mediated by excess IL-1 signaling. In contrast to the critical role of TNF at the onset of IL-1Ra−/− arthritis, no role was evident after onset of disease. This finding, and the TNF-independent joint pathology after IL-1 overexpression, indicates that IL-1 can act independent of TNF on joint inflammation, cartilage depletion and bone erosion.

098 ACTIVATION OF THE TLR3 SIGNALLING PATHWAY AND INTERFERON REGULATORY FACTOR 3 BY IKB KINASE-EPSILON IN RHEUMATOID ARTHRITIS

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Aim: Initiation and perpetuation of rheumatoid arthritis (RA) might involve activation of innate immune responses in individuals with increased immunoreactivity. We hypothesise that anti-viral gene expression in RA synovium is induced and regulated by the IKK-related kinase, inducible IKK (IKKi or IKKε), and its downstream target, interferon regulatory factor 3 (IRF). This kinase has been implicated in the activation of the anti-viral response initiated by toll-like receptor 3 (TLR) activation, resulting in RANTES and IFNβ production.
Methods: Fibroblast-like synoviocytes (FLS) and synovial tissue (ST) were derived from RA and osteoarthritis (OA) tissue at total joint replacement surgery. Synovial tissue kinase assay was performed using IKKε immunoprecipitates and c-Jun substrate. FLS were stimulated and evaluated by western blot for polyclonal P-IKKε (T501), IRF3, phosphorylated IRF3 (P-IRF3), RANTES, and IFNβ. FLS were infected with adenoviral dominant negative (DN) IKKε or Lac Z control overnight followed by Western blot for P-IRF3, RANTES, and IFNβ. Real time Q-PCR analysis was performed DNIKKε infected human FLS and murine IKKε knockout FLS stimulated with TLR ligands to evaluate the contribution of IKKε to transcription of relevant genes.
Results: Western blot demonstrates higher levels of P-IKKε in RA compared with OA tissue. In vitro kinase assay using IKKε immunoprecipitated from tissue lysate revealed that IKKε from RA lysates has greater c-Jun phosphorylating activity. Western blot analysis demonstrates the presence of downstream IRF3 and P-IRF3 protein in synovial tissue. In addition, P-IRF3 is increased in RA compared with OA synovium, confirming activation of the anti-viral signaling pathway. To evaluate the mechanisms of IRF3 activation, cultured FLS were stimulated with TLR agonists and cytokines. TLR3 ligation by poly (I-C) induces phosphorylation of IRF3. IRF3-regulated gene expression of both RANTES and IFNβ protein was increased by poly (I-C) from 4–24 hr in FLS. Because IKKε can potentially phosphorylate IRF3, FLS were infected with adenoviral DNIKKε and assessed for P-IRF3 and downstream gene expression. DNIKKε inhibited phosphorylation of IRF3, as well as RANTES and IFNβ production, confirming the role of IKKε in this pathway. FLS from IKKε −/− mice were used to confirm the functional relevance of IKKε on downstream IFN stimulated gene expression. Expression of RANTES, a key component of the anti-viral response, was decreased in LPS or poly (I-C) treated knockout FLS compared with wild type FLS. IFNβ gene expression, decreased slightly and showed variability between cell lines.
Conclusion: These data demonstrate that anti-viral responses are activated in RA synovium. IKKε and IRF3 are phosphorylated in RA synovial tissue. TLR agonists, poly (I-C) and LPS, induce IRF3 phosphorylation and IFN-stimulated gene expression in FLS. Components of the anti-viral response are partially dependent on IKKε, such as IRF3 activation and subsequent RANTES and IFNβ production.

Matrix, bone, and cartilage biology

099 FCγ RECEPTORS DIRECTLY MEDIATE CARTILAGE BUT NOT BONE DESTRUCTION: UNCOUPLING OF SEVERE CARTILAGE DAMAGE FROM JOINT INFLAMMATION

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Aim: Murine antigen induced arthritis (AIA) is characterised by Immune complex dependent chronic joint inflammation and severe cartilage and bone destruction. Recently we demonstrated that activating FcgRI and III and inhibiting FcgRII are crucial in mediating cartilage destruction during murine antigen-induced arthritis. Now we studied the relation between synovial inflammation and concomitant occurrence of cartilage and bone erosion at conditions of variable inflammation in FcgR−/−.
Methods: AIA was induced by injecting of methylated BSA (mBSA:60 mg) into the right knee joint of various FcgR knockout mice and wildtype (WT) controls previously immunized with mBSA/FCA. Histology of total knee joints was taken at day 7 after arthritis induction. Joint inflammation was scored in the joint cavity and synovial layer. Cartilage destruction was measured as chondrocyte death and surface erosion. Bone destruction was determined on 10 different well-defined spots of the knee joint section.
Results: In the absence of the inhibiting FcgRII (FcgRII−/−), joint inflammation at day 7 was significantly higher (infiltrate 93% and exudate 200% higher) when compared to WT controls. Both cartilage and bone destruction were significantly elevated (100% and 156% respectively), suggesting that activating FcgR may be involved in both cartilage and bone destruction. However, when arthritis was induced in knee joints of mice lacking activating FcgRI and III (but not FcgRIIb), cartilage destruction was completely absent, whereas bone erosion was not significantly different from WT controls. Joint inflammation was comparable between the two groups, indicating that activating FcgR are crucial in mediating cartilage, which occurred uncoupled from joint inflammation, whereas bone destruction followed the degree of inflammation. This tendency was again observed in mice lacking all three FcgR. Of great interest, joint inflammation was significantly elevated (infiltrate and exudates, 100% and 188% respectively) in FcgRI/II/III−/− knee joints, due to inefficient clearance of immune complexes. Although joint inflammation was much higher in the FcgRI/II/III−/− knee joints, cartilage destruction was lowered by 92% whereas in contrast bone erosion was raised by 200%, again implying that the amount of cellular influx is correlated to bone erosion. Immunolocalisation of Cathepsin K, a crucial mediator of osteoclasts showed a strong correlation with the amount of inflammation. Cathepsin K expression by osteoclasts is not directly dependent on FcgR expression and activation.
Conclusion: Activating FcgR are crucial in mediating cartilage destruction independent of joint inflammation. In contrast, FcgR are not directly involved in bone erosion. Indirectly, the presence of immune complexes can drive joint inflammation and bone erosion.

100 BONE MORPHOGENETIC PROTEINS ARE CHONDROPROTECTIVE IN COLLAGEN INDUCED ARTHRITIS

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Aims: Increasing evidence suggests a role for bone morphogenetic proteins (BMPs) in joint homeostasis, destruction and remodeling. We studied the expression and activation of BMPs and downstream signaling molecules in collagen induced arthritis (CIA), a mouse model of systemic

auto-immune arthritis. We also evaluated the effect of local or systemic overexpression of noggin, a BMP antagonist, on CIA.
Materials and Methods: CIA was induced by allo-immunisation with chicken type II collagen (CII) emulsified in complete Freund adjuvant on day 0, followed by booster injection of CII on day 21. In expression studies, onset of arthritis was synchronised with LPS injection at day 25. Knees were collected for RNA extraction (4 sets of 3 pooled knees) and for immunohistochemistry (IHC) at day 0, 20, 27, 33, 40 and 47. BMP expression levels were determined by real time quantitative PCR. IHC for P-Smad was performed on paraffin-embedded sections. For local overexpression of Noggin, knees and ankles of mice were injected intra-articularly with 106 pfu Noggin or control adenovirus 14 days after immunisation. Mice were sacrificed at day 30. Noggin was systemically overexpressed by injection of 300 μg pcDNA-plasmid containing Noggin or empty vector in m. tibialis anterior at day 19. Mice were sacrificed at day 50. Incidence and severity of arthritis were assessed clinically and histologically (exsudate, infiltration, pannus formation, cartilage and bone destruction). Cartilage loss was quantified using safranin O staining and digital image analysis.
Results: Expression of different BMPs (BMP2, 3, 4, 6, 7 and GDF5) was upregulated in arthritic knees time-dependently. Peak expression was found at day 20. The BMP/Smad pathway was activated in the arthritic joint. In the initial disease stages P-Smad staining was seen in the lining layer, subsequently spread towards the subintima and was later found in the invading pannus. Local and systemic overexpression of Noggin did not affect incidence and severity (clinical and histological) of CIA synovitis. However, cartilage destruction, determined by digital image analysis, was significantly increased in mice treated locally with a BMP antagonist, suggesting that loss of BMP renders the cartilage more vulnerable.
Conclusions: These data provide further evidence that BMP signaling is involved in the balance between tissue destruction, homeostasis and remodeling. Shifting the balance in BMP signaling, by local over-expression of Noggin, increases cartilage damage. The absence of a detectable difference in synovitis characteristics suggests a complex regulatory network in the synovium and pannus with potential compensatory mechanisms.

101 LONG TERM EFFECTS OF CHONDROSPHERES ON CARTILAGE LESIONS AS INVESTIGATED IN THE SCID MOUSE MODEL

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Background: Different mechanic traumata often ressult in articular cartilaginous lesions. Due to the bradytrophic character of cartilage, these lesions can hardly be repaired by the organism itself. Autologous chondrocyte transplantation (ACT) using cultured chondrocytes in monolayers has been one attempt to compensate for the structural defect, however, results are not always convincing.
Methods: Human chondrocytes were isolated from 5 patients undergoing articular arthroplastic surgery and cultured for 3–5 weeks in monolayer followed by condensing single chondrocytes to so-called chondrospheres. In addition, chondrospheres were aggregated to 7–12 fusion aggregates. To examine the long-term stability, the chondrospheres were implanted within inert sponges or embedded in periostal tissue using the SCID mouse co-implantation model. In a second approach, the integrative capacity of the chondrospheres was investigated by implanting them into lesions in human cartilage specimens and co-implanting the constructs in the SCID mouse model. Mice were sacrificed after 4, 12 and 24 weeks, and the implants were paraffin-embedded. For evaluation, 5 μm slices were generated, and HE and safranin O staining as well as immunohistochemistry using anti-S100, anti-collagen I and II antibodies were performed and analysed using a semi-quantitative score (0: expression of 0–25% of cells; 1: 26–50%, 2: 51–75%, 3: 76–100%). ''Cohesion'' of the chondrosphere with the (native) cartilage matrix is given in percent of adhering surface.
Results: With respect to long-term stability, chondrospheres showed a preserved chondrocytic morphological appearance; immunohistochemically, a high collagen II production could be detected. Concerning integration into cartilaginous lesions, after 4, 12 and 24 weeks, 7, 9 and 6 implants of chondrospheres and human cartilage, respectively, could be evaluated. Histological examination revealed the following expression scores:

4 weeks: Collagen I: 2.4; Collagen II: 2.0; S100: 2.3; Safranin O: 1.2; EvG: 2.1, Cohesion: 3.7; chondrocyte morphology: 1.7.

12 weeks: Collagen I: 1.6; Collagen II: 3.0; S100: 2.8; Safranin O: 2.2; EvG: 2.6, Cohesion: 4.6; chondrocyte morphology: 2.9.

24 weeks: Collagen I: 2.4; Collagen II: 2.9; S100: 2.4; Safranin O: 2.6; EvG: 2.7, Cohesion: 6.1; chondrocyte morphology: 2.9.

Conclusion: Taking together, these data suggest that cultured chondrospheres are able to restore and conserve their phenotype for at least 24 weeks under in vivo conditions. Moreover, chondrospheres adhere to superficial cartilage defects and appear to produce a cartilaginous extracellular matrix, which fuses which native cartilage thus generating autologous cartilage substitute.

102 A NOVEL ROLE FOR SOCS3 IN CARTILAGE DESTRUCTION VIA INDUCTION OF CHONDROCYTE DESENSITISATION TOWARDS IGF-1

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Introduction: Chondrocyte desensitisation towards its main anabolic factor, Insulin-like growth factor (IGF)-1 is an important contributing mechanism of cartilage destruction in arthritis. In previous studies, we identified Interleukin(IL)-1 as the responsible inflammatory cytokine causing suppression of proteoglycan (PG) synthesis and IGF-1 non-responsiveness of chondrocytes in murine arthritis models. The IL-1 induced suppression of PG synthesis is mediated by nitric-oxide (NO), however, the mechanism causing IGF-1 non-responsiveness is still unknown.
Aim: To explore the role of suppressor of cytokine signaling (SOCS)3 in the IL-1 induced IGF-1 desensitisation of chondrocytes.
Methods: Chondrocyte responsiveness to IGF-1 was assessed by 35S-sulphate incorporation into proteoglycans, via aggrecan mRNA expression using RT-QPCR, or insulin-receptor substrate (IRS)-1 tyrosine phosphorylation (western blot). IGF-1 desensitisation of patellar chondrocytes was studied in zymosan-induced arthritis (ZIA). IGF-1 desensitization was induced in patellar cartilage explants or H4 chondrocyte cell line, exposed to IL-1a. SOCS3 protein expression was assessed by immunohistochemistry or by westernblot analysis of protein extracts. The role of SOCS3 in IGF-1 signaling was elucidated by adenoviral overexpression.
Results: Exposure of murine articular cartilage to IL-1 almost completely blocked the IGF-1 induced PG synthesis and this effect could not be prevented by the addition of inducible nitric-oxide (iNOS) inhibitors (NIO, LNMMA). This effect of IL-1 induced IGF-1 desensitisation was also obtained with patellar cartilage from iNOS−/− mice, revealing the involvement of a secondary IL-1 induced factor, other than NO. For this, we studied the role of SOCS proteins as these are negative feedback regulators of signal transducer and activators of transcription (STAT) and are implicated in many growth factor responses. In H4 chondrocytes we showed that IL-1 significantly up-regulated SOCS3 transcription and protein synthesis. In contrast, IL-18 was unable to induce SOCS3 expression and failed to induce chondrocyte IGF-1 desensitisation. In addition, histology taken from arthritic knee joints also revealed high expression of SOCS3 in the chondrocytes. Next, we studied the effect of adenoviral overexpression of SOCS3 and obtained direct evidence that SOCS3 can inhibit IGF-1-mediated cell signaling by reducing IRS-1 phosphorylation. The inhibition of IGF-1 induced aggrecan synthesis by forced SOCS3 expression was comparable to the observed IL-1 effect on these H4 chondrocytes.
Conclusion: In the present study, we show that IL-1 induced SOCS3 expression is a novel mechanism of IGF-1 desensitisation in chondrocytes, and together with NO it can contribute to cartilage damage during arthritis.

103 A TNF α MEDIATED IL-1β CASCADE IN OSTEOARTHRITIC CHONDROCYTES IS REPRESSED BY POLYSULPHATED BETA-CYCLODEXTRINS

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Aim: In osteoarthritic (OA) articular chondrocytes, the anabolic IGFRI/insulin-like growth factor 1 (IGF-1) pathway was shown to be overruled by the catabolic effects of the IL-1/IL-1RI pathway. As a consequence less of the produced extracellular matrix macromolecules may persist in the CAM of the OA chondrocytes. The influence of polysulphated β-cyclodextrin on this TNFα-mediated auto/paracrine IL-1/IL-1RI pathway has been evaluated.
Methods: Chondrocytes were isolated from both visually intact and fibrillated femoral condyle cartilage of human knee joints and cultured separately in gelled alginate to maintain their differentiated phenotype. During 1 week of culture, the cells were exposed to 5 μg/ml of 2,3,6-trisulphate-β-cyclodextrin. Additionally, normal chondrocyte cultures with and without 2,3,6-trisulphate-β-cyclodextrin were exposed to 180 pg/ml TNFα during the last 48 hours. In a second experiment chondrocytes from 4 additional donors were incubated at day 5 of culture with 5 μg/ml of resp. 2,6-di-O-methyl-3-sulphate-beta-cyclodextrin, 2,6-di-O-pentyl-3-sulphate-beta-cyclodextrin, 6-monodeoxy-6-monoamino-2,3-disulphate-beta-cyclodextrin, 2,3-di-O-methyl-6-sulphate-beta-cyclodextrin and 2,3,6-trisulphate-β-cyclodextrin during

5 days. The release of IL-6 in the culture media was used as a variable reflecting the auto/paracrine IL-1β activity of the cells in different experimental conditions. The aggrecan content in the ECM was assayed by conventional ELISA.
Results: Chondrocytes from OA regions secreted 2 to 3 times more IL-6 in the supernatant media compared to cells from intact cartilage. The cells of intact cartilage showed a fourfold increase in IL-6 release when exposed to 180 pg/ml of TNFα. 5.0 microg/ml of β-cyclodextrin polysulphate caused a 50% decrease of the IL-6 concentration in the supernatant of normal and osteoarthritic chondrocytes and a 30% decrease in TNFα primed chondrocytes. Monosulphated cyclodextrins failed to affect chondrocyte aggrecan synthesis and IL-6 secretion. Di- and trisulphated cyclodextrin, by contrast improved chondrocyte aggrecan synthesis by 30% and significantly repressed IL-6 secretion.
Conclusion: As a result of an auto/paracrine TNFα/IL-1 loop, OA cells secreted significantly more IL-6 than normal chondrocytes. 2,3,6-trisulphate-β-cyclodextrin repressed the synthesis of IL-6 in both normal and OA chondrocytes during the first week of culture. The additonal experiments show that polysulphated, but not monosulphated cyclodextrins, inhibit the autocrine IL-1 pathway, reflected by a decreased IL-6 synthesis, and promotes aggrecan synthesis. This indicates the reduction of ECM degradation and thus the normalisation of chondrocyte metabolism in homeostatic OA cartilage. Considering IL-1β as the major auto/paracrine cytokine inducing catabolic processes in cartilage, its downregulation by β-cyclodextrin polysulphate may be a novel approach in enhancing repair of osteoarthritic cartilage.

104 BILATERAL OSTEOPOROSIS OF TIBIAL METAPHYSIS IN RAT ANTIGEN INDUCED ARTHRITIS OF RIGHT KNEE JOINT: A MICRO-COMPUTED TOMOGRAPHY STUDY

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Background and Objectives: Rats with antigen-induced arthritis (AIA) exhibit marked inflammation and damage of periarticular bone. Joint swelling and bone structure were analysed at both tibial metaphysis to assess whether methotrexate or dexamethasone any influence on the feature of AIA. Microcomputed tomography (micro-CT) allows true three-dimensional structural analysis of bone.
Methods: AIA was induced in right knee joints of female Lewis rats. The animals were divided into four treatment groups (n = 8) and one healthy control group (n = 8). Rats in the treatment groups received intraperitoneal injections of MTX (3 mg/kg/week), dexamethasone (0,25 mg/kg/day, 9x day 0–17) or phosphate-buffered saline (PBS, 8x day 0–17). Progression of AIA was monitored by measuring of joint swelling and comparison with the left knee joint. Micro-CT analyses with a resolution of 25 μm were performed at the secondary spongiosa of right tibial metaphysis on day 20. In four animals of each treatment group analyses were also performed on left tibial metaphysis at the same areas. Morphologic parameters determined were bone volume (BV/TV), bone surface (BS/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp).
Results: In animals with AIA BV/TV, Tb.N, Tb.Th of the tibial metaphysis were significantly lower and BS/TV and Tb.Sp significantly higher as in healthy controls at the side of the arthritic joint. The same effect was found at the opposite side. Among different treatment regimes with MTX and dexamethasone there were no differences in all of these morphologic bone parameters compared with PBS-treated AIA.
The inflammatory activity assessed by measurement of joint swelling was significantly reduced in the dexamethasone-treated group compared with PBS-treated AIA. MTX had no influence on joint swelling.
Conclusions: Micro-CT analysis revealed for the first time bilateral bone loss at tibial metaphysis in the monoarthritic animal model of AIA. MTX and dexamethason had no influence on arthritis-induced periarticular and systemic bone loss in AIA of rats. Only dexamethasone leads to a significant reduction in joint swelling.

105 INFLUENCE OF TNF-α AND IL-1β ON MATRIX DEGRADATION BY SYNOVIAL FIBROBLASTS IN AN IN VITRO CARTILAGE/PANNUS MODEL

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Background: Aggressive synovial fibroblasts (SF) at the cartilage/pannus-junction play an important role in joint destruction and inflammation in rheumatoid arthritis (RA). They locally express tissue-destructive enzymes (e.g. several MMPs and aggrecanase) and contribute to joint destruction by digesting the extracellular matrix. In vivo, SF and chondrocytes in the articular cartilage are stimulated by soluble cytokines and/or cell-cell contact with infiltrating inflammatory cells.
Objective: To analyse the influence of stimulation on SF from (RA) with the proinflamatory cytokines TNF-α and IL-1β in an in vitro coculture system with bovine articular cartilage (BC) explants.
Methods: Standardised BC discs were embedded in a 48 well plate containing agarose. RA-SF were either i) pre-treated with TNF-α (10 ng/ml), IL-1β (5 ng/ml) or the combination of both cytokines for 24 h and then cocultured with BC; or ii) permanently treated with the respective cytokines during coculture (2 weeks). Destruction of the BC was monitored by measuring the release of sulfated glycosaminoglycan into the supernatant (Dimethylenblue-binding assay) and by safranin-O staining of histological sections. Overall MMP activity in the supernatant was quantified using a fluorescence assay with a broadly MMP-specific peptide substrate. Classification of the individual MMP family members and discrimination of latent and active forms was achieved by gelatine/casein zymography.
Results: Cytokine pre-treatment of SF did not affect proteoglycan depletion and MMP activity. Continuous stimulation with IL-1β or IL-1β/TNF-α, but not TNF-α alone, significantly increased proteoglycan depletion from the cartilage (up to 2.5fold) both in the BC monoculture and the coculture with SF, showing a similar magnitude in both cultures. In addition, significantly higher levels of overall MMP activity after continuous stimulation with IL-1β, TNF-α or IL-1β/TNF-α were observed in both the BC monoculture and the coculture with SF. Furthermore, the overall MMP activity in the coculture with SF showed up to 2 fold higher levels compared with the BC monoculture. Permanently treated BC monocultures and cocultures with SF , but not pre-treated or untreated samples showed an increased MMP 2, MMP 9 and caseinolytic activity.
Conclusion: Permanent stimulation with TNF-α, IL-1β and TNF-α/IL-1β resulted in an increased cartilage degradation and MMP activity in both BC monocultures and cocultures with SF.
Comparison of the 2 different culture systems with the respective controls and with each other may allow to distinguish the relative contribution of the BC and the SF to the cartilage degradation.

106 SERUM OSTEOPROTEGERIN LEVELS AND BONE CHANGES IN DIFFUSE IDIOPATHIC SKELETAL HYPEROSTOSIS AND ANKYLOSING SPONDYLITIS

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Introduction: Diffuse idiopathic skeletal hyperostosis (DISH) and ankylosing spondylitis (AS) are different diseases characterised by spinal ossifications. Heterotopic ossifications in DISH probably result from abnormal activity of osteoblasts in bone-ligament insertion associated with insufficient local inhibition of mineralisation. No changes in markers of bone turnover showing new bone formation have been found.
Several molecules from the TNF family of ligands and receptors play an important role in bone remodelling. Osteoprotegerin (OPG) competitively binds receptor activator of nuclear factor kappa B ligand (RANKL) thereby inhibiting differentiation and activation of osteoclasts. Conflicting results have been reported regarding the OPG serum levels in AS. There has not been any report on the RANK-OPG-RANKL system in DISH.
Aim: To compare serum OPG levels in patients with DISH and AS; explore the correlation with inflammatory activity, bone remodelling markers and bone mineral density (BMD).
Methods: Forty three AS patients (age 18–75), 71 patients with DISH (age 46–84) and 116 healthy controls (20–80) were investigated. OPG was detected in serum samples by ELISA. BMD was measured by bone densitometry. Serum levels of osteocalcin, bone alkaline phosphatase and urine deoxypyridinoline were detected by EIA. Activity of AS was evaluated by BASDAI, ESR and CRP.
Results: Mean levels of OPG were higher in DISH (5.3+−2.03 pmol/l) in comparison with AS (3.4+−1.4 pmol/l) and control group (4.1+−1.95 pmol/l, $p<0.05$). There was a correlation of OPG with increasing age and after adjustment the difference between groups was lost. OPG levels positively correlated with BMD in lumbar spine ($p<0,05$) in AS patients, even after adjustment for age. Serum OPG levels in AS patients with isolated axial involvement correlated with BASDAI ($p=0.043$). Patients with AS and with osteoporosis had lower levels of OPG (2.4+−0.65 pmol/l) in comparison with those with normal BMD (3.6+−1.3 pmol/l, $p=0.004$). Serum osteocalcin levels were higher in AS patients (10.4+−6.4 ug/l) than in DISH

(7.2+−4.3 ug/l, p=0.001), however, after adjustment for age this correlation was no longer found.
Conclusion: When interpreting serum OPG and osteocalcin levels the age has to be taken into account. Formation of heterotopic ossifications is not associated with increased systemic levels of OPG. Low OPG levels may be implicated in the pathogenesis of osteoporosis in AS.
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107 MCP-1 DEPENDENT OSTEOCLASTOGENESIS CAN BE RESCUED BY TNF-α

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Rheumatoid arthritis (RA) is characterised by osteoclast mediated local and systemic bone loss.

Chemokines such as monocyte chemoattractant protein-1 (MCP-1) might be essential not only for the recruitment of haematopoetic osteoclast precursors into the inflamed synovium but also for osteoclast differentiation. We therefore investigated the role of MCP-1 in osteoclastogenesis under inflammatory as well as non-inflammatory conditions in vitro.

Murine spleen derived monocytes were cultured with 30 ng/ml MCSF and 50 ng/ml RANKL to induce osteoclastogenesis. After 5 days of cell culture, TRAP staining was performed. TRAP-positive mononuclear cells (osteoclast precursors) and multinuclear (osteoclasts) were counted.

In this in vitro assay addition of blocking antibodies against murine MCP-1 was able to inhibit osteoclast formation dose dependently up to 60% clearly indicating that MCP-1 plays an important role in osteoclastogenesis. Due to the fact that blocking of MCP-1 did not affect the numbers of osteoclast precursors and late addition of the antibodies resulted in the same amount of osteoclast blockade, we conclude MCP-1 likely affects osteoclast differentiation and not the proliferation of osteoclast precursors.

To mimic osteoclastogenesis under inflammatory conditions, we added TNF-α to the in vitro osteoclast formation assay. As previously shown, addition of TNF-α resulted in enhanced numbers of osteoclasts. Surprisingly anti MCP-1 antibody, not even in the highest concentration (10 μg/ml), was able to block osteoclast formation under these inflammatory conditions.

The concentration of MCP-1 in culture supernatants as measured with ELISA did not differ significantly among inflammatory (TNF-induced) and non-inflammatory conditions (mean±SEM: 458.5±13.45 vs. 491.0±45.78 pg/ml respectively). Furthermore, addition of blocking anti-MCP-1 antibodies resulted in a dose dependent reduction of MCP-1 levels under both conditions. This clearly indicates that the lack of inhibiting effect of anti MCP-1 antibodies in TNF-α stimulated cell cultures was not due to increased MCP-1 levels. To detect potentially different levels of CCR2, the receptor binding MCP-1, we used western blot analysis from cell lysates stimulated with or without TNF-α. Again we could not observe any expression regulating effect of TNF-α, indicating that the failure of anti MCP-1 antibodies to inhibit osteoclastogenesis under inflammatory conditions was not due to a down regulation of CCR2 by TNF-α.

Taking together we conclude, that MCP-1 is an important mediator for osteoclastogenesis in the absence but not in the presence of TNF-α.

108 SERUM TYPE II COLLAGEN EPITOPE MEASUREMENT PREDICTS RADIOGRAPHIC PROGRESSION DURING TREATMENT WITH ANTI-TNFα THERAPY

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Introduction: Prevention of radiographic progression in inflammatory arthritis is limited by the lack of predictive biomarkers for joint destruction. The serum level of the type II collagen propeptide CPII correlates with type II collagen synthesis, and is elevated in RA. The collagen cleavage neo-epitope C2C is specific for the destruction of type II collagen by the collagenases MMP-1, MMP-8 and MMP-13. The ratio of C2C/CPII is increased in osteoarthritis and has been shown to correlate with cartilage destruction. The current study assessed whether serum C2C and CPII levels can predict radiographic progression in a group of patients with rheumatoid arthritis (RA) and psoriatic arthritis (PsA) during biological therapy.
Methods: RA (n=40) and PSA (n=11) were assessed before and at 1 and 3 months timepoints after commencement of anti-TNFα therapy. DAS28 score, ESR and HAQ were measured at each assessment. CPII and C2C were measured by ELISA. The association between the changes in DAS28, ESR, HAQ and the ratio CPII/C2C after one and three months with radiographic progression at one year was analysed using SPSS statistical software.
Results: Modified Sharp score after 12 months increased by 2.6±0.8 (mean±SE, p<0.01) Sharp Units (SU) with no difference between disease categories. Increased radiographic progression (>2SU) was associated with a greater decrease in CPII/C2C after one month of therapy (−1.71 vs −0.63, p=0.006, ANOVA). Stepwise linear regression analyses for radiographic progression using the 0–1 month change in CPII/C2C, DAS28, HAQ and ESR as independent variables showed a significant relationship for CPII/C2C at one month (p=0.008). No relationship was seen using the three month changes in independent variables.
Conclusion: Measurement of the CPII/C2C ratio may more closely reflect cartilage changes in RA through measurement of both the synthesis and destruction of type II collagen. Changes in the ratio of CPII/C2C reflect radiographic outcome very early after commencement with anti-TNFα therapy. The targeted use of biological therapies using cartilage biomarkers as an early response marker may aid clinicians and improve patient outcomes.

Endothelial and fibroblasts biology

109 INDUCTION OF A PROFIBROTIC PROFILE VIA FGFR3 IN SYSTEMIC SCLEROSIS

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Fibroblast growth factor receptor 3 (FGFR3) belongs to a family of 4 different FGF receptors. Alterations of signaling via FGF receptors have been linked to skeletal dysformations and some forms of cancer.

Differential screening of the dermis of SSc patients and healthy controls revealed an overexpression of FGFR3 in systemic sclerosis (SSc). The overexpression of FGFR3 was confirmed by real-time PCR and in-situ hybridisation. The upregulation of FGFR3 mRNA and protein was also detected in cultured SSc fibroblasts. The expression of other members of the FGF receptor family was not altered in SSc patients. Only one isoform of FGFR3, FGFR3 b, was upregulated in vivo. FGF-9, a ligand for FGFR3, was also overexpressed in the skin of SSc patients. To characterise the effects of the overexpression of FGFR3 in SSc patients, mice with constitutive signaling via FGFR3 (FGFR3G374Rneo-) and mice lacking FGFR3 (FGFR3G374Rneo+), were employed. Analyses of dermal fibroblasts from these animals with Affymetrix array revealed an induction of profibrotic mediators such as MCP-1, CTGF, endothelin-1, endothelin receptor B and IL-4 receptor α upon FGFR3-activation. Increased amounts of collagens were produced by FGFR3G374Rneo- fibroblasts in vitro. Stimulation of human dermal fibroblasts from SSc patients and healthy controls with FGF-9, the ligand found to be overexpressed in SSc, confirmed the induction of profibrotic mediators and collagens upon FGFR3 signaling. Of note, SSc fibroblasts were more responsive to FGF-9 than controls.

These data suggest that in SSc patients FGFR3 and its ligand FGF-9 are upstream of several profibrotic mediators such as MCP-1, CTGF, endothelin-1, endothelin receptor B and IL-4 receptor α, which are known to play key roles in the pathogenesis of SSc. Therapeutic strategies targeting FGFR3 might therefore inhibit the efficiently the accumulation of extracellular matrix components by preventing simultaneously the upregulation of multiple profibrotic mediators.

110 SYSTEMIC SCLEROSIS (SSC) FIBROBLASTS REDUCE ANGIOGENESIS IN HEALTHY MICROVASCULAR ENDOTHELIAL CELLS (H-MVEC) BY MATRIX METALLOPROTEINASE-12 (MMP-12) DEPENDENT CLEAVAGE OF UROKINASE RECEPTOR (CD87, U-PAR)

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Background: Endothelial damage and defective neoangiogenesis are hallmarks of SSc. We previously showed that in SSc-MVEC the impaired angiogenesis is associated to the cleavage of u-PAR by MMP-12.
Objectives: To evaluate the effects of SSc fibroblasts on healthy MVEC (H-MVEC) angiogenic potential, particularly on 1) proliferation; 2) chemoinvasion; 3) angiogenesis in vitro; 4) integrity of u-PAR. Methods: The effects of SSc fibroblasts (S-F) were evaluated incubating H-MVEC, in basal conditions or induced by urokinase (u-PA) 250 ng/ml or VEGF 20 ng/ml, with the conditioned medium from healthy (H-CM) and S-F

(S-CM). We evaluated: proliferation by cell count; chemoinvasion by the Boyden chamber assay; angiogenesis by the capillary morphogenesis assay; the domains of u-PAR by western blotting; MMP-12 expression by western blotting and RT-PCR; role of MMP-12 performing experiments in the presence/absence of anti–MMP-12 mAb.
Results: In H-MVEC, H-CM had no effects on proliferation, chemoinvasion and capillary morphogenesis while S-CM caused a significant inhibition of all these features, even when induced by u-PA or VEGF, that was reverted by the concomitant treatment with anti–MMP-12 mAb. S-CM, but not H-CM, induced the cleavage of u-PAR. MMP-12 in S-F versus H-F was higher expressed and protein levels were higher, especially for the ratio released in the culture medium. Inhibition of MMP-12 prevented u-PAR cleavage.
Conclusion: S-F conditionate H-MVEC microenvironment, secreting high levels of MMP-12, that induce u-PAR cleavage, impairing their angiogenic properties and counteracting the response to angiogenic factors. Then, SSc fibroblasts alterations might be considered as an early feature of SSc that, by u-PAR cleavage, can impair H-MVEC angiogenesis, contributing to the pathogenesis of endothelial damage and to the so called desert-like pattern of the terminal forms of diffuse SSc.

111 ALTERATIONS OF TNF-α/TNF-RECEPTOR 1 INDUCED MAP KINASE SIGNAL TRANSDUCTION MAY CONTRIBUTE TO THE DESTRUCTIVE PHENOTYPE OF RA-SFB

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Aim: TNF-α is a major inductor of the pro-inflammatory/pro-destructive functions of rheumatoid arthritis (RA) synovial fibroblasts (SFB). Its effects are mediated via two receptors, the TNF-receptor-1 (TNFR1) and TNF-receptor-2 (TNFR2). Although the signal transduction of TNF-α has been extensively studied in RA- and osteoarthritis (OA)-SFB, activation of the individual signal transduction pathways by each TNF-receptor has not been characterised. In the present study, RA-SFB were compared to OA-SFB concerning the TNF-α/TNF-receptor1/2-induced signal transduction of p38 MAP kinase, Erk kinase, and Jun kinase and their role in the induction of pro-destructive MMP-1 secretion.
Methods: For signal transduction analysis, RA- and OA-SFB (beginning of 2nd passage) were stimulated with TNF-α or agonistic anti-TNFR1/TNFR2 mAbs for 10 min. Phosphorylation of signal transduction molecules was analysed with phospho-specific mAbs against p38 MAP kinase, Erk kinase, and Jun kinase using western blotting. For analysis of pro-destructive MMP-1 secretion, cells were stimulated with TNF-α or agonistic anti-TNFR1/TNFR2 mAbs. p38 MAP kinase, Erk kinase, and Jun kinase were inhibited by pre-incubation of the cells with the specific inhibitors SB203580, U0126, or SP600125, respectively. Supernatants of the stimulated cells were collected after 24 h and analysed for MMP-1 concentration by ELISA.
Results: RA- and OA-SFB comparably expressed TNFR1/TNFR2 by flow cytometry. A significantly higher phosphorylation of p38 MAP kinase and Erk kinase was observed in RA- and OA-SFB compared to non-stimulated cells following 10 min stimulation with TNF-α or agonistic anti-TNFR1 mAb. Interestingly, Jun kinase phosphorylation was significantly induced only in RA-SFB, but not in OA-SFB following TNF-α/TNFR1 stimulation. MMP-1 secretion was significantly induced in RA- and OA-SFB following TNF-α/TNFR1 stimulation. Inhibition of Erk kinase had no significant influence on MMP-1 secretion in RA- and OA-SFB. In contrast, p38 MAP kinase inhibition significantly reduced MMP-1 secretion in OA-SFB, but not in RA-SFB. Strikingly, Jun kinase inhibition significantly reduced MMP-1 secretion in RA-SFB, but not in OA-SFB.
Conclusions: In early-passage RA- and OA-SFB, TNF-α-induced signal transduction is predominantly mediated by TNFR1. Strikingly, specific Jun kinase phosphorylation following TNF-α/TNFR1 stimulation only occurs in RA-SFB. Differential induction of the Jun kinase pathway and a partial resistance of RA-SFB to p38 MAP kinase inhibition may contribute to increased production of pro-destructive effector molecules and represents a potential therapeutic target in RA.

112 ANTIFIBROTIC POTENTIAL OF PROTEASOME BLOCKADE

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Tissue fibrosis results when dysregulation of extracellular matrix (ECM) turnover favors deposition of collagen and other ECM proteins over degradation. Fibrosis may then lead to organ dysfunction and pathology as observed in systemic sclerosis (SSc). In the present study we investigated the anti-fibrotic properties of proteasome blockade. A dose- and time-dependent reduction in type-I collagen and TIMP-1 production was observed in normal fibroblasts exposed to proteasome inhibitors (PI). In the same culture conditions, MMP-1 protein and the collagenolytic activity on type I collagen was increased. The steady-state mRNA levels of COL1A1, TIMP-1, and MMP-1 paralleled protein levels. These effects were dominant over the pro-fibrotic properties of TGF-β and observed with fibroblasts generated from normal and SSc skin. PI decreased type I collagen mRNA levels with kinetics similar to those observed with DRB, a specific RNA polymerase II inhibitor, thus indicating transcriptional inhibition. Of interest, PI induced c-Jun phosphorylation and c-Jun nuclear accumulation. The specific N-terminal Jun-kinase inhibitor SP-600125 selectively abrogated c-Jun phosphorylation and, in a dose-dependent fashion, the upregulated synthesis of MMP-1 induced by PI. Finally, PI did not affect fibroblast viability. Thus, the coordinated downregulation of collagen and TIMP-1 and upregulation of MMP-1 renders proteasome blockade an attractive strategy for treating conditions as SSc, characterised by excessive fibrosis.

113 INDUCTION OF IL-16 EXPRESSION IN SYNOVIAL FIBROBLASTS BY TGF-β AND LAMININ-1 DOES NOT REQUIRE ACTIVATION OF THE CELLS BY TNF-α OR IL-1β

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Aim: In rheumatoid arthritis (RA), synovial fibroblast-like cells (SF) contribute significantly to articular inflammation. They express elevated levels of cytokines and chemoattractant factors, including IL-16. Therefore IL-16 regulatory pathways were investigated in SF.
Materials and Methods: Early passage RA-SF were expanded and activated by addition of cytokines and chemokines and by incubation on plates coated with matrix proteins. Regulation of IL-16 mRNA was investigated by quantitative RT/PCR. The IL-16 cytokine was detected in SF supernatants by ELISA and by chemotactic activity using PBMC as target cells.
Results: Addition of TGF-β to SF elevated IL-16 mRNA signals significantly, VEGF and RANTES did not influence IL-16 mRNA levels, whereas IL-1β or TNF-α reduced IL-16 mRNA amounts in SF. Incubation of synovial fibroblasts on laminin-1 significantly raised IL-16 mRNA in SF whereas attachment to laminin-10, type I collagen, and other matrix proteins had no such effects. Addition of TGF-β to SF attached to laminin-1 raised the IL-16 mRNA expression in a dose dependent manner and statistically significant. The IL-16 induction was associated with extra cellular signal related kinase (ERK) signalling. The secretion of the IL-16 cytokine by RA-SF was induced by IL-1β, IGF-1 and TGF-β and enhanced in all cases by laminin-1 co-signalling.
Conclusion: Attachment of SF to laminin-1 in presence of TGF-β triggers IL-16 mRNA expression. This pathway represents a novel IL-16 induction mechanism and does not require addition of the pro-inflammatory cytokines IL-1 or TNF.

114 A-SAA INDUCES CHEMOKINE EXPRESSION, CELL INVASION AND CYTOSKELETAL DISASSEMBLY IN RA SYNOVIAL TISSUE, IN PART THROUGH THE PI3 KINASE AND NFκB PATHWAYS

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Introduction: Rheumatoid arthritis (RA) is a chronic inflammatory arthritis characterised by progressive joint destruction, damage and disability. Serum amyloid A (A-SAA) is an acute phase protein with cytokine-like properties and we have demonstrated high levels in serum and joints of patients with RA. Furthermore A-SAA can induce cell adhesion and angiogenesis in vitro, processes that enhance leucocyte migration, which is essential for persistent tissue infiltration in the inflammatory joint.
Aim: To identify mechanisms involved in A-SAA-induced cell migration in RA.

Methods: Chemokine expression (CXCL8, CCL2), cell invasion, neutrophil transendothelial cell migration and cytoskeletal assembly/disassembly were examined in RA synovial fibroblast (RASFC), human microvascular endothelial cells (HDVEC) and in RA whole tissue synovial explant cultures using Flow cytometry, ELISA, vertical invasion assays, fibronectin coated transwell migration assays and F-actin immunofluorescent staining.
Results: A-SAA (10 μg/ml) at physiological levels significantly enhanced expression of CXCL8 ($p<0.05$) and CCL2 ($p<0.05$) in primary RASFC and HDVEC. Furthermore, A-SAA induced CXCL8 and CCL2 production in RA synovial explant cultures, where the tissue architecture and cell-cell contact is maintained. CXCL8 and CCL2 expression was significantly enhanced by A-SAA at 3h reaching maximum levels at 48 h, an effect that was comparable to stimulation by interleukin-1 beta (IL-1β) and tumour necrosis factor-alpha (TNF-α). A-SAA significantly stimulated a 2.8 fold increase over basal in HDVEC cell invasion ($p<0.05$), an effect that was greater than that of TNF-α and IL-1β In addition, A-SAA induced a dramatic increase in neutrophil transendothelial cell migration following 16h incubation, from a basal level of 11×104 migrating cells to 65×104 in response to A-SAA. Furthermore, using RASFC and HDVEC, A-SAA induced disassembly of the actin cytoskeleton and dramatically induced filopodia formation compared to vehicle control, which displayed intact actin stress fibres. Finally, we demonstrated that SAA-induced CCL2 expression in RA synovial explant cultures is mediated through the phosphatidyl-inositol 3 kinase (PI3K) and NFkB pathway, since addition of an inhibitor to PI3K (LY294002) and NFkB (NAC), decreased A-SAA induced synovial explant CCL2 expression by 80%.
Conclusion: These data suggest that A-SAA is a key pro-inflammatory mediator in the RA joint and may represent a novel target for therapy.

115 SURVIVIN AND NOTCH SIGNALLING IN BLOOD VESSEL MATURATION AND SYNOVIAL HYPERPLASIA IN RHEUMATOID ARTHRITIS

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Background: Inflammatory arthritis is characterised by increased angiogenesis, cell recruitment and aberrant proliferation of synovial fibroblasts. This process critically depends on endothelial cell (EC) survival mechanisms to preserve the integrity of synovial blood vessels.
Aim: To determine blood vessel maturity and expression, regulation and activity of downstream survival signalling pathways (Survivin and Notch) in microvascular EC (MVEC), rheumatoid arthritis synovial fibroblasts (RASFC), and RA whole tissue synovial explant cultures.
Methods: Blood vessel maturity was examined using dual immunofluorescence staining for factor VIII (EC) and alphaSMA (pericytes) in synovial tissue, pre- and post- IL-1 ra therapy alone or in combination with anti-TNFα, and was assessed using confocal microscopy. Expression of Survivin and Notch signalling components, at baseline and following stimulation with angiogenic factors VEGF, Ang 1 or Ang 2 were determined by SDS-PAGE and RT-PCR in MVEC, RASFC and RA synovial explant cultures. Furthermore, their effect on CBF-1-dependent Notch pathway activation was determined by luciferase reporter analysis or RT-PCR in MVEC and RASFC.
Results: The majority of synovial blood vessels in RA (89.43%+7.29%) have recruited pericytes, suggesting they continue to undergo active maturation. Blood vessel maturity was not significantly affected following treatment with IL-1ra alone or in combination with anti-TNFα therapy. We demonstrated expression of Notch receptors and survivin in synovial perivascular and lining regions. Treatment with VEGF and Ang 2 increased Notch 3 IC and Notch 4 IC protein expression by 2.25- and 3.52-fold respectively in RA synovial explant cultures. VEGF increased survivin mRNA expression in MVEC over 72 h, with a maximal increase of 1.4-fold at 48 h. In addition, VEGF and Ang 2 stimulation increases survivin protein expression by 1.6- and 1.4-fold respectively in MVEC, and transactivates CBF-1-dependent promoter activity, with the most significant increases being 2- and 5.5-fold at 8 h and 24 h post-stimulation respectively. We also demonstrated the presence of the Notch receptors, ligands and the CBF-1-dependent Notch target genes, HRT-1, -2 and -3 in RASFC using immunohistochemistry, western blotting and RT-PCR, suggesting they may play a role in RASFC survival and invasion. Furthermore, we have shown that VEGF increases Notch 1 and HRT-1 mRNA expression (two and six fold respectively). A three fold increase in Notch 1 mRNA expression has also been demonstrated following stimulation with Ang 1.
Conclusion: These data demonstrate synovial blood vessels are undergoing maturation, and suggest that the Notch and survivin signalling pathways play a critical, complimentary role in preserving blood vessel maturity in inflammatory arthritis. In addition, we establish the presence of the Notch signalling pathway in RASFC, and for the first time establish CBF-1-dependent Notch pathway activity in these cells, which may contribute to RA synovial hyperplasia.

116 GELSOLIN DEFICIENCY PROTECTS MICE FROM BLEOMYCIN INDUCED PULMONARY FIBROSIS

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Aim: Idiopathic pulmonary fibrosis (IPF, also known as cryptogenic fibrosing alveolitis) is a progressive and lethal pulmonary disease of unknown etiology, characterised by the histological pattern of usual interstitial pneumonia (UIP). Gelsolin is an actin-binding/severing protein that modulates cytoskeletal rearrangements and which has been implicated in caspase-3 mediated apoptosis. Its deregulated expression has been correlated with a wide range of tumors and was recently shown to play a prominent role in the pathogenesis of rheumatoid arthritis. To dissect the potential role of gelsolin in the development of IPF, gelsolin null mice ($gsn^{-/-}$) were injected intravenously with BLM, together with wt littermates.
Materials and Methods: For the induction of the disease we utilised the Bleomycin (BLM) animal model of IPF which shares many clinical features with the human disease and has provided valuable insights into the pathogenetic mechanisms that govern disease activation and perpetuation.
Results: Decreased levels of gelsolin expression in IPF were detected, through expression profiling (utilising DNA microarrays and real time RT-PCR) of disease progression in the BLM animal model. As expected, wt mice developed progressive, heterogeneous pulmonary inflammation and fibrosis that led to the collapse of air spaces. In sharp contrast, $gsn^{-/-}$ mice exhibited no signs of the disease, maintaining normal lung architecture. The observed protection from IPF correlated with reduced neutrophil infiltration, due to the reduced secretion of the major neutrophil chemoattractant KC, as well as to the reduced chemotactic responses of gelsolin null neutrophils. Moreover, preliminary reciprocal bone marrow transplantation experiments (wt ↔ $gsn^{-/-}$) attributed the observed protection from IPF to gelsolin deficiency in stromal cells, thus highlighting the role of KC secretion from the epithelial cells. In turn, the reduced KC secretion correlated with reduced numbers of apoptotic (TUNEL positive) epithelial cells and decreased caspase-3 enzymatic activity in $gsn^{-/-}$ lungs, thus suggesting a role for gelsolin in the regulation of epithelial apoptosis.
Conclusion: *gsn* deficiency was shown to protect mice from BLM-induced IPF, most likely through the inhibition of epithelial apoptosis and subsequent KC secretion and neutrophil chemotaxis.

Mesenchymal stem cells

117 BONE MARROW MESENCHYMAL STEM CELL PROPERTIES IN PATIENTS WITH RHEUMATOID ARTHRITIS

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Objective: The use of bone marrow (BM) mesenchymal stem cells (MSCs) in cartilage and bone repair is currently under investigation. The aim of the study is to evaluate the number, the functional and molecular characteristics and the osteogenic and chondrogenic differentiation potential of BM MSCs in patients with rheumatoid arthritis (RA).
Methods: We have studied 22 RA patients and 22 age- and sex-matched healthy controls. The BM mononuclear cells (BMMCs) were isolated from posterior iliac crest aspirates and the MSCs were expanded according to the standard protocol. MSCs were characterised by their immunophenotypic characteristics ($CD45^-$,$CD14^-$,$CD34^-$, $CD90^+$,$CD73^+$,$CD44^+$,$CD29^+$,$CD105^+$,$-CD146^+$) and their adipogenic (Oil red O stain and aP2 and PPAR-γ expression), osteogenic (ALP/Von Kossa stain and ALP and CBFA1 expression), and chondrogenic (Masson and Alcian blue stain and Collagen II and Aggrecan expression) potential. The frequency of MSCs in the BMMC fraction

was evaluated by a limiting-dilution assay (LDA). The functional characteristics of MSCs were studied by evaluating (a) their clonogenic potential using a standard colony forming unit-fibroblast (CFU-F) assay and enumerating the CFU-Fs/100MSCs plated through passages (P), (b) their proliferative potential time-course by using the MTT assay and evaluating the cell doubling time (2^n = cells counted/cells plated) in each passage, and (c) the production of TNFα, TGFβ, LIF, VEGF and SDF in MSC culture supernatants time-course. The Wnt5A and Fz5 expression in MSCs at day 0 ($CD45^-$/$GlycoA^-$ BMMCs) was evaluated by a semi-quantitative RT-PCR.
Results: RA patients displayed normal number and immunophenotypic characteristics of BM MSCs. The chondrogenic and osteogenic potential of patient MSCs did not differ from the respective of the controls as was evaluated by the collagen II, aggrecan and ALP, CBFA1 mRNA expression, respectively, by means of a semi-quantitative RT-PCR. Compared to healthy controls, however, patient MSCs displayed impaired CFU-F forming potential time-course ($P<0.01$; P0-P6) as well as impaired proliferative capacity. This was demonstrated by the MTT assay at P1 ($P<0.01$) and the cell doubling time time-course ($P<0.01$; P1-P7). No statistically significant difference was found between RA patients and healthy controls in the levels of inhibitory (TNFα, TGFβ) and stimulatory (LIF, VEGF, SDF) cytokines in MSC culture supernatants at P2, P4, P6. Patient MSCs, however, at P0 displayed lower Wnt5A and Fz5 mRNA expression compared to the controls ($P=0.0095$ and $P=0.0208$, respectively).
Conclusion: RA patients display normal number and immunophenotypic characteristics of BM MSCs. Patient MSCs display normal chondrogenic and osteogenic capacity but defective proliferative and clonogenic potential. Since Wnt5A and Fz5 has been shown to display a prominent role in the proliferation and differentiation process of BM stem cells, the down-regulation of these molecules in RA MSCs may have a pathogenetic role in the above abnormalities.

118 SYNOVIAL INFLAMMATION REDUCES CHONDROGENIC POTENTIAL OF RESIDENT MESENCHYMAL STEM CELLS

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Background: There is clinical evidence that adequate suppression of joint inflammation with methotrexate and anti-TNF leads to spontaneous joint repair with healing of bone erosions.[1] In our previous study, we showed that in the synovial fluid the numbers of mesenchymal stem cells (MSCs) and their chondrogenic potential were lower in RA compared to OA.[2] Here we hypothesised that the inflammatory environment of the diseased joint had a direct effect on the function of MSCs resident in synovial tissue.
Methods: Synovial tissue biopsies reflecting differing degrees of inflammation were taken during diagnostic arthroscopy from 10 patients (6 RA and 4 OA) and a visual analogue score (VAS) was used as a measure of macroscopic joint inflammation. One biopsy was used for functional assessment of MSC activity and the other one for immunohistochemistry. Following tissue digestion with collagenase, synovial cells were grown in DMEM/10% FCS and passage 3 cultures were tested in quantitative trilineage mesenchymal differentiation assays. Immunohistochemistry and semi-quantitative analysis (SQA)[3] were used to assess synovial inflammation. CD68 and CD3 staining indicated macrophage and T-cell infiltration, and TNF-α, IL-6, IL-1-α and IL-1ra were used to evaluate synovial cytokine milieu.
Results: A significant negative correlation was found between VAS score and MSC chondrogenesis ($R=-0.705$, $P=0.020$) whereas synovial MSC osteogenic and adipogenic capacities remained unaffected. Analysis of a parallel biopsy revealed no significant correlation between chondrogenesis and inflammatory indicators such as lining layer thickness, numbers of T-cells or macrophages, or levels of TNF-α or other cytokines. This suggested that VAS score could be a better indicator of gross joint inflammation state than synovial markers. In general, chondrogenesis of cultures derived from RA patients (1.8 ± 1.2 GAG/pellet, VAS 33 ± 2) was over two fold lower than that of OA patients (3.8 ± 1.4 μg GAG/pellet, VAS 8 ± 2).
Conclusions: These data indicate that inflammation affects the chondrogenic capacity of resident synovial tissue MSCs in arthritis. These findings have implications for the understanding of biology of MSCs in the inflammatory environment and highlight the importance of controlling inflammation as a pre-requisite for MSC cell therapy in arthritis. Also, VAS score could be considered as a potency indicator to predict cartilage repair potential of autologous synovium-derived MSCs.

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119 PROINFLAMMATORY CYTOKINES REDUCE THE EXPRESSION OF THE SOX9 CHONDROGENIC FACTOR IN MESENCHYMAL STEM CELLS

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Rheumatologist recognise the potential value of using joint derived autologous mesenchymal stem cells (MSCs) for regenerative medicine in OA and RA. However, the fact that MSCs in the joint has been directly exposed to inflammation disease has not been given sufficient attention. In addition, the effect of the inflammatory environment in which MCS would be re-implanted has not been considered. We have shown that chondrogenesis was qualitatively diminished in MSCs derived from the synovial fluid of RA patients compared to OA.[1] Here we first used patient derived MSCs and second, an in vitro model using cytokines to investigate the effect of inflammation on the ability of MSCs to differentiate by quantifying the expression of three essential transcription factors (TFs), Sox9, Runx2 and PPAR-γ responsible for chondro- osteo- and adipogenesis, respectively. We isolated and expanded MSCs using standard methods from the synovial fluid of RA (n = 6) and OA (n = 3) patients and used healthy bone marrow MSCs as controls (n = 6). The expression of Sox9, Runx2 and PPAR-γ was measured by real time PCR. The expression of Runx2 was comparable in control, OA and RA MSCs. PPAR-γ was reduced only in RA. Sox9 expression was slightly increased in OA compared to control but reduced 10-fold in RA ($P<0.05$). Using a surrogate measure of inflammation in the synovial fluid (WCC), we observed a trend toward Sox9 expression being inversely correlated with inflammation levels in RA (n = 3). The effect of TNF-α, TGF-β1, IFN-γ and IL-4 on the expression of Sox9, Runx2 and PPAR-γ was further investigated on control MSCs. Cultures were treated for 24 hours with the different cytokines. Our pilot data show that TNF-α has a profound negative effect on the expression of all 3 TFs. TGF-β1 is a trigger of chondrogenesis and accordingly increased the expression of Sox9 and reduced the expression of Runx2. INF-γ only had a negative effect on the expression of Runx2 whereas IL-4 affected both Sox9 and Runx2 but not PPAR-γ. These findings support recent evidence that TNF-α and IL-1 can directly down-regulate the expression of all three mesenchymal lineage TFs.[2–4] Our data also suggest that cytokine(s) other than TNF-α are likely to be involved in the preferential down-regulation of chondrogenesis in RA. Controlling joint inflammation with anti-TNF is associated with the down-regulation of inflammation associated parameters (including bone damage)[5] and would therefore be necessary, but may not be sufficient to allow MSCs to undertake cartilage repair in RA.

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Genetics, genomics, and proteomics

120 RHEUMATOID ARTHRITIS SUBTYPES IDENTIFIED BY GENOMIC PROFILING OF PERIPHERAL BLOOD CELLS: ASSIGNMENT OF A VIRAL RESPONSE SIGNATURE IN A SUBPOPULATION OF PATIENTS

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Aim: Rheumatoid arthritis (RA) is a heterogeneous disease with unknown aetiology. Evidence exists that the underlying pathology varies between patients. The aim of the current study is to evaluate whether heterogeneity in RA is reflected in the gene expression profile of peripheral blood cells.
Methods: We analysed whole blood gene expression profiles of 35 RA patients and 15 age- and sex-matched healthy controls, using microarrays with a complexity of ~20.000 unique genes. Genes that were significantly different expressed between RA patients and healthy controls, and among RA patients were selected using Statistical Analysis of Microarrays (SAM). Gene expression data was further analysed by hierarchical clustering and geneset analysis to obtain a biologcal interpretation of the differentially expressed genes.

Results: Statistical analysis of expression data identified 577 genes with a significantly different expression level in RA patients compared to healthy controls. A most prominent set of correlated genes consisted of known interferon-induced genes. Application of gene set analysis algorithms identified processes involved in an anti-viral host response program. Subsequent comparison of the expression profiles of the RA patients with a viral-induced expression program revealed that a subgroup of RA patients showed striking similarities with a viral response. The RA patients with a viral infection-associated expression program showed higher levels of the IFN-response genes, and increased serum anti-CCP antibody levels.
Conclusion: Genomic profiling of peripheral blood cells of RA patients reveals an immune defense host response gene expression profile, mimicking an anti-viral host response, that defines a subgroup of RA patients characterised by an increased autoreactivity against citrunillated proteins.

121 THE NOVEL TRANSCRIPTION REGULATORY POLYMORPHISM -43T>C IN THE 5′-FLANKING REGION OF THE SLC19A1 GENE AND METHOTREXATE TRANSPORT

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Background/Aim: Methotraxate (MTX) is the cornerstone therapy of rheumatoid arthritis (RA) due to the high clinical experience compared to other disease-modifying anti-rheumatic drugs (DMARDs). However, the response of RA patients to MTX shows high variability. The reduced folate carrier (RFC-protein, SLC19A1-gene) has central role in the uptake and intracellular accumulation of folates. In this respect, we investigate whether SLC19A1 genetic variations are related with the RA patient efficacy or toxicity to MTX therapy.
Patients and Methods: One hundred seventeen unrelated patients with RA were enrolled in this study. Genomic DNA was analysed by polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP). Unusual SSCP patterns were characterised by direct sequencing of the PCR products and subsequently restriction assays were established for the identified genetic changes. Western blot analysis of human RFC plasma membrane protein was performed in respect of the identified SLC19A1 genotypes. Patient response to MTX was evaluated using DAS-28, ACR-20 and ACR-50 scores.
Results: No mutation was not found in our patients, but 3 polymorphic variants were identified: the novel -43T>C and the previously reported 80G>A (R27H) and 696C>T (P232P). The wild type alleles of the 3 polymorphisms were in strict linkage disequilibrium. Western blot analysis revealed that the -43T>C polymorphism is functional and is associated with low RFC protein expression levels. The -43T>C is located in the putative AP1 (Activator Protein-1) transcription factor consensus sequence in the 5′-flanking gene region of the SLC19A1 gene and therefore this nucleotide substitution probably affects AP1 binding to its enhancer, SLC19A1 gene expression and finally MTX transport inside the cell.
Conclusion: The wild type allele of polymorphism -43T>C could potentially positively affect patient response to MTX therapy, which is known to be controlled by multiple transport systems and factors.

122 ACTIVE IMMUNISATION AGAINST TNFα PROTECTS HUMAN-TNFα TRANSGENIC MICE FROM ARTHRITIS

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Aim: Targeting TNFα has demonstrated its high efficacy in rheumatoid arthritis, although some concerns remain such as the high cost and side effects. Currently available treatments use passive immunisation with anti-TNFα antibodies or administration of the receptor. We explored the feasibility and the efficiency of a therapeutic vaccination in a model of rheumatoid arthritis driven by TNFα.
Methods: Human (h) TNFα was complexed to keyhole limpet hemocyanin (KLH) with glutaraldehyde to form hTNFα-KLH heterocomplex. Active immunisation was performed by 3 intramuscular injections of hTNFα-KLH (priming with 30 μg; boosts with 30 and 15 μg at resp days 7 and 29) in hTNFα transgenic mice (Taconic). Mice (10 per group) were treated either with hTNFα-KLH, or with hTNFα-KLH and methotrexate (MTX) (1 mg/kg). Control mice were treated with either KLH or saline. Treatments started when mice were 5 weeks old. Mice were clinically evaluated twice a week. Anti-hTNFα levels and neutralising activity were measured by a sandwich ELISA and with L929 cells, respectively. Cytokine levels were measured by ELISA. Histology of paws was performed at the 9th week after priming.
Results: Clinical arthritis was dramatically improved in hTNFα vaccinated mice with no additive effect of MTX (maximal arthritic scores: resp: 0.9 ± 0.5 and 1.0 ± 0.3, vs 11.5 ± 4.2 and 9.0 ± 1.4 in PBS and KLH control groups, $p<0.001$). Histological analysis revealed a clear cut effect of hTNFα vaccination on inflammatory synovitis (resp: 0.16 ± 0.09 and $0.24\pm.0.09$ in hTNFα-KLH without or with MTX, vs 1.30 ± 0.09 and 1.53 ± 0.21 in PBS and KLH control groups, $p<0.01$); similar results were observed on articular destruction (resp: 0.08 ± 0.03 and 0.21 ± 0.12 in hTNFα-KLH without or with MTX, vs 0.71 ± 0.11 and 1.10 ± 0.23 in PBS and KLH control groups, $p<0.01$). All immunised mice developped high levels of anti-hTNFα antibodies. High levels of hTNFα were detected in control mice sera, contrasting with vaccinated mice that displayed no detectable hTNFα levels. Mouse TNFα or IL-6 was not detected in any serum. Longer term experiments are in progress showing a sustained inhibition of arthritis in this model four months after immunisation.
Conclusions: Active immunisation with hTNFα-KLH inhibits clinical arthritis, histological synovitis and joint destruction, and allows high levels of anti hTNFα antibodies that abolished the detection of TNFα in sera. Further experiments are in progress to explore the long term kinetics of TNFα inhibition and the tolerance of this anti-TNFα strategy.

123 THE HLA-DRB1 SHARED EPITOPE ALLELES ARE PRIMARILY A RISK FACTOR FOR ANTI-CITRULLINATED PROTEIN ANTIBODIES AND NOT FOR THE DEVELOPMENT OF RHEUMATOID ARTHRITIS

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Although the pathways leading to the emergence and perpetuation of the destructive immune response in rheumatoid arthritis (RA) are not elucidated, it is generally accepted that genetic risk factors contribute significantly. The most prominent and well-known genetic risk factor is the HLA-region, particularly the HLA-alleles encoding for a common amino acid sequence in the third hypervariable region of the DRb1 molecule. However, the biological pathways explaining the association of HLA with RA are still unclear, as the nature of the immune response responsible for joint destruction is not known. Recently it became clear that antibodies directed against citrullinated proteins (ACPA) are highly specific and predictive for RA, that they are associated with disease progression and that the antigens recognised by these antibodies are present in the inflamed joint.

We now show, by taking advantage of a well-described inception cohort consisting of 440 early RA patients, that SE-encoding HLA alleles only associate with RA that is characterised by the presence of ACPA, and not with ACPA negative disease. These results were confirmed with the help of linkage analysis in sib pairs of 341 families with well-established RA. In contrast, HLA-DR3 only associates with ACPA negative disease. These data are important as they show that distinct genetic risk factors are associated with distinct phenotypes of RA, thereby indicating that different pathogenetic mechanisms are underlying ACPA positive and negative RA.

Analysis of the clinical evolution in combination with genetic and serologic risk factors of patients prone to develop RA allows insight in the factors that are associated with progression towards RA. SE-encoding alleles are a risk factor for RA development in patients with undifferentiated arthritis (UA). Intriguingly, when UA-patients were stratified for the presence of ACPA, the SE-alleles did not contribute to RA-development in either ACPA positive or negative UA. Together, these data constitute an important refinement of the long-known association between HLA and RA by indicating that the SE alleles do not associate with RA as such, but rather act as immune response gene for the development of ACPA.

124 A NEW SUBTRACTIVE PHAGE DISPLAY METHOD TO DISCOVER AUTOANTIBODY TARGETS

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It is still unknown why autoimmune patients produce antibodies directed against self-antigens. A recent hypothesis proposes that unusually modified proteins or protein complexes trigger the autoimmune response

and apoptotic cells have been proposed to represent a reservoir of modified autoantigens. When the clearance of apoptotic cell remnants is somehow disturbed, one can imagine that the apoptotically modified structures become exposed to the immune system. We believe that the immune system identifies apoptotic modifications as ''cryptic'' epitopes and that this might lead to the initiation of autoimmune responses.

Antibody phage display is an established technique to produce recombinant antibodies against all kinds of antigens. We have established a new subtractive selection method that facilitates the selection of recombinant antibodies against those epitopes that are uniquely or predominantly present in only one of two complex protein mixtures. Application of this new technology can help to elucidate differences between diseased and healthy tissue or cell extracts on a proteomic level.

Our selection method comprises two parts. First, antibody phage display libraries generated from the complete antibody repertoire of autoimmune patients are subtractively panned on the two cell or tissue extracts. Second, the selected phage pools, enriched for phages that are differentially reactive with these extracts, are applied to western blots of two-dimensional IEF/SDS-PAGE gels containing proteins from both cell extracts in parallel to further select for differentially reactive phages. Phages recognising protein spots exclusively present on one of the two western blots are eluted and amplified.

We successfully used antibody phage display libraries derived from RA (rheumatoid arthritis), SLE (systemic lupus erythematosus) and SSc (systemic sclerosis) patients in this new two-step subtractive selection procedure and obtained single chain variable fragment antibodies to several epitopes that are present in apoptotic but not in non-apoptotic cell extracts. These recombinant antibodies were used for immunoaffinity purification of the target proteins, which were subsequently identified by mass spectrometry as hnRNP C, PSF/p54nrb, and U1-70K. The U1-70K protein is a well-described autoantigenic protein in SLE overlap syndrome. U1-70K is a component of the U1 snRNP complex, and is cleaved early during apoptosis. Fully consistent with the selection procedure, the anti-U1-70K antibody appeared to be strongly reactive with the 40 kD apoptotic fragment of U1-70K, whereas it is hardly or not reactive with the intact U1-70K protein.

We conclude that the new subtractive (auto)antibody selection method allows the selective isolation of (auto)antibodies from phage display libraries, which target epitopes that are only expressed under certain physiological conditions.

125 A GENOMICS APPROACH TO ANALYSE TNF-α BIOACTIVITY IN RHEUMATOID ARTHRITIS SYNOVIAL TISSUE

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Introduction: Rheumatoid arthritis (RA) is a chronic inflammatory disorder affecting the synovial joints. Tumour necrosis factor (TNF)-α plays an important role in synovial inflammation and cartilage and bone erosion in RA, and is thus an important target for therapy. However, despite the beneficial effects of TNF blockade a significant proportion of the patients do not respond at all indicative for the existence of different pathways that contribute to disease in RA.
Aim: In this study, we aim to identify rheumatoid synovial tissues that express features of TNF-α-bioactivity.
Methods: FLS (n = 4) were cultured for 4–6 passages, and were stimulated for 6 hours with 10 ng/ml TNF-α. Gene expression profiles from RA synovial tissues (n = 16) and cultured FLS were analysed using cDNA microarrays. Stastical Analysis for Microarrays (SAM) was used to demonstrate significance. Subclassification of tissues and FLS was performed by hierarchical cluster analysis. To confirm our microarray data and to determine amounts of TNF-α mRNA, we performed Real Time quantitative PCR.
Results: A TNF-α signature was created by comparing the gene expression profile of fibroblast-like synoviocytes (FLS) from RA patients that were stimulated for 6 hours with TNF-α with that of unstimulated FLS. Genes that were increased at least four fold (p-value <0.05 based on SAM) were included in the TNF-α signature. This signature was subsequently used to perform hierarchical cluster analysis on gene expression profiles of the RA synovial tissues. This analysis allowed us to separate patients on the basis of the presence or absence of a TNF-α signature in their synovium.
Conclusion: Rheumatoid arthritis synovial tissues show differential involvement of TNF-α bioactivity. Further research is needed to find out whether the presence of synovial TNF-α bioactivity is indicative for responsiveness to anti-TNF therapy.

126 DEK GENE AND SUSCEPTIBILITY TO RHEUMATOID ARTHITIS

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Aim: Recently Brintnell *et al* presented evidence for a novel rheumatoid arthritis (RA) susceptibility locus on chromosome 6p that is independent of HLA-DRB1 gene. Fine mapping of the region under the linkage peak led to the identification of a single nucleotide polymorphism (SNP) in 3′UTR of *DEK* gene (rs3476G/A) that weakly associates with susceptibility to RA (P = 0.07). DEK is an abundant nuclear protein bound to chromatin that is able to change DNA topology, functions in chromatin remodeling and could modulate gene expression. Presence of autoantibodies to DEK protein in juvenile rheumatoid arthritis (JRA); systemic lupus erythematosus (SLE), RA and systemic sclerosis indicate possible involvement of DEK in autoimmune diseases. We investigated association of rs3476G/A with RA in Dutch population and its role in RA susceptibility and severity.
Materials and Methods: 913 RA patients and 560 control individuals were genotyped for the 3′UTR *DEK* SNP using restriction length fragment polymorphism (RLFP). CCP and shared epitope (SE) status were known for 583 and 571 RA patients respectively . Genotyping data from all RA patients were combined for analysis.
Results: Comparison of cases and controls did not reveal a significant difference in genotype frequencies of rs3476 between the groups (P = 0.37, table 1). Allele frequencies were also comparable in RA patients and controls (77.3 vs. 79.1% for the G allele and 22.1 vs. 20.9% for the A allele, P = 0.26). Stratification of RA patients according to anti-CCP status showed marginal association of rare allele with CCP positive RA.
Conclusion: No association of *DEK* rs3476G polymorphism was found in large population of Dutch RA patients. Our data indicate that previously reported association of rs3476G allele with RA is most likely, due to a small sample size analysed, a false positive finding.

Abstract 126 Distribution of genotypes and allele frequencies of *DEK* rs3476 in cases and controls

	Genotypes (%)				Allele freq (%)			
	GG	GA	AA	p	G	A	OR (95% CI)	p
Controls	62.5 (350)	33.2 (186)	4.3 (24)		79.1			20.9
RA patients	60.6 (553)	33.5 (306)	5.9 (54)	0.37	77.3	22.1	1.11 (0.92–1.34)	0.26
Shared epitope								
Positive	61.8 (240)	34.5 (134)	3.6 (23)	0.82	77.3	22.7	1.11 (0.89–1.39)	0.35
Negative	60.3 (105)	35.1 (61)	4.6 (8)	0.88	77.9	22.1	1.08 (0.80–1.45)	0.6
Anti - CCP								
Positive	56.8 (192)	35.8 (121)	7.4 (25)	0.07	74.7	25.3	1.28 (1.02–1.62)	0.03
Negative	62.7 (154)	31.8 (78)	5.3 (13)	0.78	78.8	21.2	1.02 (0.78–1.33)	0.88

127 ASSOCIATION OF FCGR HAPLOTYPES WITH EARLY RHEUMATOID ARTHRITIS AND FCGR3B WITH ARTICULAR EROSIONS AT BASELINE

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Background: Rheumatoid arthritis (RA) is an autoimmune disease that is thought to arise from a complex interaction between multiple genetic factors and environmental triggers. We have recently demonstrated an association between a FCGR3A-FCGR3B haplotype and susceptibility to RA and the development of nodules.[1] The FCGR genes encode the FcγR and FcγRIIIa is expressed on macrophages and NK cells. The

FCGR3A-158V allele has an increased affinity for IgG-containing immune complexes. FcγRIIIb is expressed on neutrophils with the NA1 and NA2 isoforms differing by four amino acids in the membrane-distal Ig-like domain. This alters the glycosylation pattern and NA2 homozygotes display reduced phagocytosis. We have now extended this study to further elucidate whether this association is also observed in an inception cohort of RA patients and have used the presence of erosions at baseline as a marker of early articular severity.
Methods: An inception cohort of 448 RA patients from the Yorkshire Early Arthritis Register (YEAR) were recruited into this study, 100 of whom had clearly defined joint erosions at diagnosis. RA patients and 267 healthy blood donor controls were genotyped for the FCGR3A-158F/V polymorphism using SSCP[2] and the FCGR3B-NA1/2 polymorphism by ARMS PCR.[1] Genotype and haplotype frequencies and linkage disequilibrium (D′) were estimated and a model-free analysis performed to determine association with RA.
Results: Homozygosity for the FCGR3A-FCGR3B 158V-NA2 haplotype was associated with an increased susceptibility to RA (OR 2.60, 95% CI 1.3–5.1 P=0.002) in our early RA cohort compared with controls. In addition, there was a significant association with homozygosity for FCGR3B-NA2 allele and erosive disease at presentation (OR 1.8, 95% CI 1.1–2.8, P=0.02). Logistic regression analyses were then performed to assess the relative contribution of FCGR3A, FCGR3B, rheumatoid factor and the HLA-DRB1 alleles comprising the ''shared epitope'' to the presence of erosions at baseline. FCGR3B was the only significant variable (OR 1.8, 95% CI 1.2–2.8, P=0.005) in this model. We have confirmed the presence of a copy number polymorphism in FCGR3B and methods are being devised to enable copy number to be incorporated into our genotyping assays.
Conclusions: We have confirmed that homozygosity for the FCGR3A-FCGR3B 158V-NA2 haplotype is associated with an increased susceptibility to RA using our inception cohort. Perhaps more intriguing is the association between FCGR3B and early erosion development. FcγRIIIb is expressed solely on neutrophils, which are the most abundant cell type in synovial fluid. Further investigation of the role of neutrophils in RA pathogenesis is warranted.

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128 FAMILIAL AGGREGATION OF RELATED DISEASES IN FIRST DEGREE RELATIVES OF 50 CONSECUTIVE PATIENTS WITH SYSTEMIC SCLERODERMA

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Aim: The highest risk factor known for the occurrence of systemic scleroderma (SSc) is the presence of another patient with the same disease within the family. However, such families are rare. On the other hand, different autoimmune diseases (AIDs) and rheumatic diseases (RDs) may share genetic variations. The aim of this study therefore was to characterise the familial occurrence of related diseases within first degree relatives (FDRs) of 50 consecutive patients with SSc.
Material and Methods: More than 50 consecutive patients with systemic scleroderma (SSc) appearing in the ambulance and ward for diagnosis and treatment were in addition interviewed intensively (⩾ 45 min) concerning their family situation (number, sex and age of FDRs, diseases observed in the family, deaths and cause of death etc). These index patients were asked to inform their relatives about the project and to find out whether these would agree to participate themselves. Most did so and were themselves interviewed, personally or by phone. Health status of all index patients and FDRs were proven by statements in written medical reports and by details of the diagnoses. Only if all required information could be collected, the family was integrated into the final analysis.
Results: Complete families of 50 SSc patients were finally analysed. Sixteen of the SSc index patients were classified as ''familial'' (32%) and thirty-four as ''sporadic'' (68%), according to the presence or absence of other AIDs or RDs within the 325 interviewed FDRs. Most of the AIDs or RDs occurred within the FDRs in a frequency clearly above the values from published reports on prevalence in the German or European (Caucasian) population. As an example, there were 6 patients with rheumatoid arthritis, equivalent to a prevalence of 1846/100 000, i. e. 3.3-fold more frequent than expected, representing a familial relative risk (FRR) of 3.3. We observed 1 patient with SSc among 325 FDRs, indicating a FRR of 69.9 – which corresponds in the order of magnitude with published data. FRRs for SLE, MCTD, M Crohn or M Wegener were calculated to be 12.9, 11.2, 2–9 and 102, respectively.
Conclusions: FDRs of SSc patients suffer from other related AIDs and RDs more frequently than expected from prevalence data, indicating common genetic factors shared by different diseases. These may be identified within the families by further genetic analyses.
Supported by the German Network of Systemic Scleroderma.

129 CD3Z POLYMORPHISM IS ASSOCIATED WITH REDUCED EXPRESSION OF THE TCRZETA CHAIN: ENRICHMENT IN PATIENTS WITH SEVERE RHEUMATOID ARTHRITIS

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Background: The T cell receptor (TCR) is a multisubunit complex, comprising at least eight transmembrane units. The clonotypic TCRα and beta chains are responsible for antigen recognition, whilst the invariant chains of the CD3 complex and two zeta polypeptides couple antigen recognition to downstream signal transduction pathways. The zeta chain is also vital for the assembly and expression of the TCR/CD3 complex at the cell surface. TCRzeta is down-regulated in chronic inflammatory diseases such as SLE and rheumatoid arthritis (RA), leading to profound hyporesponsiveness to antigen stimulation. Using a FACS-based assay, we have identified in healthy donors a population of circulating lymphocytes expressing low levels of TCRzeta. Parallel studies have generated a haplotype and single nucleotide polymorphism (SNP) map of the human TCRzeta (CD3Z) locus. We have established a relationship between low levels of TCRzeta in peripheral blood lymphocytes and two specific SNPs (designated CD3Z7 (c.1507G>C) and CD3Z8 (c.1514A>T) in the 3′untranslated region (UTR) of the CD3Z gene in patients with SLE: heterozygosity for the minor alleles of these SNPs was associated with an increased proportion of circulating TCRzeta dim cells. We aimed to test the hypothesis that the association between CD3Z genotype and TCRzeta chain expression defined in patients with SLE could also be confirmed in healthy donors and patients with RA.
Methods: We initially established a FACS-based TCRzeta expression index reflecting 1) constitutive expression of TCRzeta in TCRzeta positive T cells and 2) the number of TCRzeta dim T cells expressed as a ratio of TCRzeta bright/dim events. Using this composite index, we compared TCRzeta expression in CD3+, CD4+, CD8+ and CD56+ lymphocyte subsets from 45 healthy donors genotyped for the CD3Z7 and CD3Z8 SNPs. TCRzeta expression was also analysed in 36 patients with severe RA requiring anti-TNF therapy. Genotyping was performed by PCR-RFLP.
Results: TCRzeta expression was reduced in all T cell subsets in donors carrying one copy of the minor allele of both SNPs; this association was true for both TCRzeta expression indices. Analysis of the 36 RA patients revealed that 36% carried at least 1 copy of the minor alleles of both SNPs compared to frequencies of 15% (for CD3Z7) and 17% (for CD3Z8) in a cohort of 962 healthy controls.
Conclusions: These data suggest that variation in expression of TCRzeta is linked to polymorphisms in the 3′UTR of the human CD3Z gene and that minor alleles of the CD3Z gene may be a marker for severe RA. As the 3′UTR of mRNA plays a key role in post-transcriptional gene regulation by affecting mRNA stability, it is feasible that reduced TCRzeta expression may occur via impaired TCRzeta mRNA stability. These findings could have important implications for understanding the genetic basis of the immune response.

130 HLA-DRB1 ALLELES SHOW A CONSERVED BACKBONE STRUCTURE VALIDATING POCKET-WISE ANALYSIS OF HLA-DISEASE ASSOCIATION: A ROUTE TO THE PROFILING OF DISEASE SPECIFIC PATHOGENIC PEPTIDES

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Aim: During antigen presentation peptides are bound to HLA molecules by anchoring amino acid residues of the antigenic peptide in pocket-like structures in the antigen binding groove. These pockets are shaped by the HLA contact residues that make up the wall of these pockets. Genetic variations in these contact residues increase or decrease affinity for peptides. Recently, we proposed that such pockets may be considered as independent entities explaining genetic epidemiological data on HLA-disease association, thus linking genetic epidemiological data with information on the 3-dimensional structure and the function of these HLA molecules. For rheumatoid arthritis such an anchor model explains the genetic epidemiological data on RA better than the shared epitope hypothesis.

In order to validate our assumption that pockets may be considered to operate independently regarding their effects on disease susceptibility we assessed whether the backbone structure of individual pockets in the HLA-DRB1 antigen binding groove changed due to substitutions in other pockets.
Materials and Methods: In order to validate the independency assumption in this approach we use in silico modelling to evaluate the effect of allelic substitutions on overall backbone structure. Published crystallographic data of HLA-DRB1 molecules were used to create 3-dimensional representations of the backbone structures of the HLA-DRB1 molecules. Positional ambiguity in each individual crystal was colour-coded at each position, and subsequently the individual structures were projected over each other.
Results: The results show a very tight fit overall, most pronounced in the beta-pleated sheeths forming the bottom of the antigen binding groove. Other regions show only minor variation, structural variation being highest in the shared epitope region. The latter coincides with maximum ambiguity in the individual crystal structures at these positions and reflects the relatively loose constraints on this area, located between two kinks in the alpha-helix.
Conclusion: Overlays of 3-dimensional structures do support the anchor model approach in which genetic variations in the different pockets can be considered to influence disease risk independently. In turn, this supports previous results of the anchor model implying a role for both HLA-DRB1 pocket 4 and pocket 1 in susceptibility to RA.

We hope that an approach which uses both pocket-wise analysis of genetic epidemiological data, and data linking pocket structure with pocket specificity may help to profile pathogenic peptide structures in autoimmune disease.

131 MOLECULAR GENETIC STUDY FOR FAMILIAL MEDITERRANEAN FEVER IN THE HELLENIC POPULATION OF GREECE AND CYPRUS

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Familial Mediterranean Fever (FMF) is a hereditary inflammatory disease with autosomal recessive transmission. Typically it presents as acute episodes of periodic fever accompanied by abdominal pain, chest pain, or joint pain. Appearance of renal amyloidosis indicates severe prognosis. About 40 mutations have been identified so far, some of them being very frequent. Molecular investigation of the Cypriot population reveals that about 1:8 is a carrier of one of four mutations, E148Q being the most frequent (1:12). Despite the high frequency of E148Q, only 9 of 91 patients carried it, supporting its mild nature. Among 186 Cypriot MEFV chromosomes analysed, the results are: V726A 28.5%; M694V 22%; F479L 20.4%; E148Q 5.4%; M694I 2.2%; R761H 3.2%; M680I 1.6%; unknown 16.7%. Mutation F479L is rather rare in other populations. Preliminary evidence suggests that this frequent, in the Cypriot population, mutation is associated with later age of onset of symptoms, the most debilitating of which is strong and frequent abdominal pain, with or without fevers and arthralgias. After investigation it was found that the patients – carriers of the MEFV mutation F479L shared a common haplotype, the same that Armenian patients - carriers of this mutation share. This findings lead to the conclusion that the common haplotypes shared by Greek Cypriots who carry mutation F479L is the result of a founder effect, the founder perhaps being of Armenian origin, as in previous historical periods there were massive waves of Armenians finding refuge in Cyprus. In a number of Hellenic samples from Greece tested, the F479L mutation was not present, whereas in a cohort of patients with childhood onset of FMF, F479L was found in only one patient in heterozygosity. In this same cohort the severe mutations M694I, M694V and *M*680*I* were highly represented, in accordance with the childhood onset of disease. Among the Greek childhood patients M694V was the most frequent (32.1%) followed by M680I (15.5%) and V726A (7.1%). It is our experience as well as the experience of others, that in a relatively high percentage of FMF patients no mutations are found in the MEFV gene, while there is no evidence for the existence of a second MEFV gene. One explanation is that unknown mutations still exist in areas of the gene that are not routinely tested, or these are patients who suffer from a similar but different disease.

Translation rheumatology and novel therapies

132 DYNAMICS OF MONOCYTE MIGRATION TO THE SYNOVIUM: DEVELOPMENT OF 99MTC-HMPAO SCINTIGRAPHY

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Background: Recent work has shown that treatment with a highly specific CCR1 antagonist results in a significant decrease in macrophage numbers in the rheumatoid synovium after only 2 weeks of oral treatment. Similarly, macrophage infiltration in synovial tissue was markedly reduced 48 hours after initiation of TNF-blockade, which could not be explained by apoptosis induction. Presumably, blocking migration and retention of inflammatory cells may be sufficient to reduce the macrophage infiltrate soon after initiation of treatment. Together, the data suggest that monocyte/macrophage infiltration of the inflamed synovium is a highly dynamic process. Therefore, we developed 99mTc-hexamethylpropylene amine oxime (HMPAO) labeled monocyte scintigraphy to evaluate the migratory behavior of monocytes in rheumatoid arthritis (RA) patients.
Methods: Eight patients with active RA (DAS 28>3.2) were included in the study. Peripheral blood was obtained (100 ml) and CD14+ cells were isolated using CliniMACS. After labeling with 99mTc-HMPAO and re-infusion, a scintigraphic scan was performed at 1, 2, 3, and 20 hours post-infusion. The scintigraphic scan was analysed for signal intensity in joints and other tissues. The number of positive joints and precise signal intensity of a single large joint were calculated, the latter being measured in counts per pixel, subtracting the signal from surrounding tissue. A correction was made for the injected dose, number of re-infused monocytes and a labeling standard, leading to a deduction of the percentage of re-infused monocytes per pixel. After two weeks this procedure was repeated.
Results: On average 19.9×106 (10.4–36.9×106) monocytes were isolated, with an isolation efficiency of 41.1%. Labeling with 99mTc-HMPAO resulted in a mean radioactivity of 211 (43–393) MBq. Re-infusion was well tolerated in all patients. The scintigraphic scans showed uptake in a number of joints in all patients, with a mean of 9 (range 1–25) positive joints. There was increased signal at all time-points, with maximal signal at one hour post re-infusion. Increased signal was also found in the lungs 1 hour after re-infusion, with subsequent decrease over time. At all time-points there was increased signal in liver and spleen. A median of 31,6×10–4 (1.55–88.4×10–4)% monocytes/pixel was found in the large joints selected for more detailed quantification. Joint signal intensity and swollen joint count were significantly correlated ($p<0.05$).
Conclusion: Synovial inflammation in RA patients is a highly dynamic process characterised by continuous influx of monocytes into the synovial compartment that can be assessed and quantitatived with a non-invasive imaging modality.

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133 REDUCED INFLAMMATION AND BONE EROSIONS AFTER LOCAL IFN-β GENE THERAPY

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Background and Objective: Interferon beta (IFN-β) has significant immunomodulatory properties and has received interest as a potentially therapeutic strategy for rheumatoid arthritis (RA). Systemic IFN-β treatment in patients with RA was not effective, probably due to pharmacokinetic issues. Therefore, we studied the potential of intra-articular IFN-β gene therapy in a proof of principle study.
Methods: Rats with adjuvant arthritis (AA) were intra-articularly injected with an adenoviral vector encoding the IFN-β gene (Ad.IFN-β). The effect on paw swelling was measured by water displacement plethysmometry. Synovial tissue of the hind paws was examined by immunohistochemistry and bone destruction was analysed using radiographs. In addition, quantitative RT-PCR was used to assess intra-articular IFN-β expression.
Results: The levels of IFN-β mRNA and protein peaked 2 days after intra-articular injection and declined thereafter ($p=0.02$ compared to control). Local delivery of Ad.IFN-β reduced paw swelling significantly ($p=0.02$). This was accompanied by a reduction in synovial inflammation ($p=0.04$).

The clinical effects in rat AA lasted up to 9 days. Strikingly, Ad.IFN-β treatment protected bone from erosion ($p=0.04$) and reduced levels of c-Cbl, Cbl-b (both signalling molecules essential for osteoclast activity, $p=0.03$ and $p=0.04$ respectively). In addition, the MMP-3/TIMP-1 ratio in the joints ($p=0.03$) was significantly decreased. Immunohistochemical analysis of the synovial tissue revealed a clear shift towards a more anti-inflammatory cytokine profile by reduced levels of IL-1β (47% compared to control, $p=0.03$) and increased levels of IL-10 (40% compared to control, $p=0.02$).
Conclusion: Local overexpression of IFN-β inhibits arthritis development and protects against bone destruction in rat AA. These findings validate IFN-β as a therapeutic molecule for intra-articular gene therapy of arthritis and stress the importance of a stable vector for transgene delivery.

134 LOCAL OVEREXPRESSION OF A TNF-BLOCKING AGENT UNDER CONTROL OF A DISEASE INDUCIBLE PROMOTER USING ADENO ASSOCIATED VIRUS TYPE 5 LEADS TO AMELIORATION OF ARTHRITIS

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Background and Objective: Intra-articular gene delivery using adeno-associated virus (AAV) vectors has great potential for the treatment of rheumatoid arthritis (RA). In a comparative study using 5 different serotypes containing a marker gene we found that direct injection of recombinant (r)AAV5 into the ankle joints of rats with adjuvant arthritis (AA) results in the highest synovial transduction, followed by rAAV2 showing markedly lower expression. Since anti-TNF strategies have proven effective for the treatment of RA, we studied the potential of intra-articular gene delivery using an rAAV5 encoding a chimeric human TNFα soluble receptor I variant (TNFR-Ig). The therapeutic gene was expressed under control of a constitutive CMV- or a disease-inducible NF-kB-responsive promoter.
Methods: Fibroblast-like synoviocytes (FLS) obtained from RA patients were transduced with rAAV5 containing the NF-κB-TNFR-Ig gene, and TNFR-Ig protein levels after stimulation with LPS were measured in culture medium by ELISA. Thereafter, the produced TNFR-Ig protein was tested in a bio-assay for its potential to block human and rat TNF-α using TNF-α sensitive WEHI-164 cells. In addition, 6.5×10^9 viral particles of rAAV5 containing the gene for TNFR-Ig and a CMV- or an NF-κB responsive-promoter (rAAV5.CMV-TNFR-Ig and rAAV5.NFκB-TNFR-Ig, resp.) or control vector were injected into the right ankle joints of rats with adjuvant arthritis (AA) on day 12 after adjuvant immunisation ($n=6$). Joints were harvested 2 weeks later. Paw swelling was measured by water-displacement plethysmometry. Joint sections were analysed by immunohistochemistry and total joints were crushed and the presence of the transgene was investigated by RT-PCR analysis.
Results: TNFR-Ig produced by FLS after rAAV5.NF-κB-TNFR-Ig transduction was proven bioactive in vitro and transcription was responsive to LPS. The affinity for human TNF-α was twice as high compared to rat TNF-α. In the rats treated with rAAV5.NFκB-TNFR-Ig reduced paw swelling was observed (25% reduction compared to control, $p<0.05$), whereas no effect was found using the CMV promoter. Transcription of the transgene was detectable in the joints injected with both constructs showing minimal differences in intensity. In addition, TNF-α and IL-1β expression was decreased in synovial tissue of rats treated with rAAV5.NFκB-TNFR-Ig (71% and 63% respectively, $p<0.05$).
Conclusion: These results show that rAAV5 may be a useful method for local delivery of cytokine or anti-cytokine therapy in RA. In addition, disease-regulated transgene expression for physiologically responsive gene therapy of arthritis might be feasible. The higher affinity for human TNF-α suggests that in RA patients the therapeutic effect may be even more pronounced than in rat AA.

135 POTENT ANTI-INFLAMMATORY, FULLY DISSOCIATIVE COMPOUND FOR GLUCOCORTICOID RECEPTOR-MEDIATED GENE REGULATION

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Aim: The glucocorticoid receptor (GR) is a transcription factor that regulates its target genes either positively, through direct binding of a GR homodimer to the promoter of target genes, or negatively by the interference of GR with the activity of other transcription factors through protein-protein interactions. Long-term use of glucocorticoid receptor (GR) agonists (corticosteroids) is significantly hampered by their adverse effects, which are believed to be mainly caused by the gene-activating properties of GR. Negative interference with the activity of transcription factors which drive pro-inflammatory gene expression (e.g. NF-kB, AP-1), greatly contributes to its desired anti-inflammatory capacities. A plant-derived compound, CpdA, with GR-binding capacities but not belonging to the steroidal class of GR-binding ligands, was tested both in vitro and in vivo for its dissociative effects.
Methods: In vitro testing was performed on fibroblast like synoviocytes (FLS) derived from patients with rheumatoid arthritis (RA), A549 human epithelial lung cells, L929sA mouse fibroblasts and TC10 mouse endothelial cells. mRNA levels of IL-6, COX-2 and GM-CSF were measured by RT- PCR, 6 hours after addition of TNF, which was added 1 hour after incubation with CpdA or dexamethasone (DEX). IL-6 concentrations were measured by ELISA. Induction of GRE-driven genes was determined by reporter gene analysis. The in vivo capacity to suppress inflammation was studied by induction of Collagen-Induced Arthritis (CIA) in male DBA/1 mice. When the first signs of arthritis appeared, mice were randomly assigned to a therapy (CpdA, PBS). Animals were sacrified after 8 days of therapy. Clinical disease severity was assessed by scoring the number of affected joints and the degree of joint swelling. Knee joints (femoropatellar and femorotibial joint) were assessed histopathologically on presence of exsudation, infiltration, bone erosion, cartilage destruction and proteoglycan depletion.
Results: In vitro testing in FLS clearly demonstrated the anti-inflammatory potential of both CpdA and DEX by repression of IL-6, COX-2 and GM-CSF mRNA levels as well as of IL-6 protein production. In sharp contrast to DEX, CpdA did not stimulate gene expression from GRE-driven reporter genes. Next, CpdA was tested in vivo in CIA. CpdA dosed at 12.0 mg/kg markedly reduced clinical severity compared to the PBS control group (median differences in clinical score between day 0 and day 8; PBS: +3, CpdA: −0.5, $p=0.014$) and likewise, the number of histologically uneffected knee joints was significantly higher in the CpdA treated group (OR = 11 (1.77–68), $p=0.006$).
Conclusion: These findings indicate that CpdA may be a lead compound of a novel class of anti-inflammatory agents with fully dissociated properties and might thus hold great potential for therapeutic use.

136 THE INCIDENCE OF FLARES OR NEW ONSET OF INFLAMMATORY BOWEL DISEASES IN PATIENTS WITH ANKYLOSING SPONDYLITIS EXPOSED TO ANTI-TNFα THERAPY

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Background: Ankylosing spondylitis (AS) and inflammatory bowel diseases (IBD) are clinically and pathogenetically linked. Infliximab and etanercept are efficacious for the treatment of patients with AS but only infliximab seems to work for both, Crohn's disease (CD) and ulcerative colitis (UC). Some case reports have recently raised concern about an increase of IBD activity in patients treated with anti-TNF agents.
Objective: To evaluate the incidence of new onset and flares of CD or UC in patients with AS treated with anti-TNF agents as compared to placebo.
Patients and Methods: Data from 4 placebo-controlled studies[1–4] with anti-TNF agents in AS (2 with etanercept and 2 with infliximab) and 1 open study[5] were analysed for the pre-study prevalence and the incidence of flares and new onset of IBD. In all studies, a history of IBD was not an exclusion criterium. The publications were carefully analysed and, in unclear cases, the investigators contacted. Here we present preliminary results of the analyses; the final results will be available during the EWRR meeting.
Results: A total history of IBD was reported in 32/755 patients (4.2%). Of those, 296 patients were exposed to etanercept and 291 to infliximab for 430 and 236 patient years, respectively. No flare of Crohn's disease (CD) but one flare of ulcerative colitis (UC) was reported during the placebo phase (95% CI: 0.02/100 – 5.3/100 patient years (py)) and no case of IBD occurred during treatment with infliximab (95% CI: 0–1.6/100 py). In contrast, 11 cases of IBD (3 cases of CD and 8 cases of UC) (95% CI: 1.3/100–4.6/100 py) were reported in patients treated with etanercept ($p=0.021$ in comparison to infliximab). When the incidence of CD ($p=0.32$) and UC ($p=0.057$) was analysed separately, this difference was no more significant. Of interest, among the 3 CD cases, 2 patients had new onset and one patient had a flare of CD, while out of the 8 UC cases, one had new onset and 7 flared.

Conclusions: New onset or flare of IBD is an overall rare event in AS patients on anti-TNF therapy. Treatment with etanercept seems to be weakly associated with reactivation or new onset of IBD in patients with AS.

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137 SAFETY AND EFFICACY OF DISCONTINUATION AND RE-ADMINISTRATION OF INFLIXIMAB AFTER LONG TERM CONTINUOUS THERAPY IN PATIENTS WITH ANKYLOSING SPONDYLITIS

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Objective: To analyse the one-year safety and efficacy of the anti-TNF agent infliximab in patients with ankylosing spondylitis (AS) after discontinuation following longterm therapy and clinical relapse, both clinically and by measuring antibodies against infliximab (ATI).
Methods: All 42 AS patients who had participated in a recently published multicenter trial with continuous infliximab therapy (5 mg/kg/6 weeks) over 3 years[1] of discontinued treatment (TP1).[2] In case of clinical relapse (as defined by BASDAI>4, TP2)), patients were reinfused with infliximab and followed up for one year (TP3). Primary outcome was the safety of the patients at TP3. Secondary outcomes were a BASDAI 50% response and fulfilment of the ASAS partial remission criteria at TP3 as compared to TP2. The concentrations of ATI (measured by ELISA) at TP2 were correlated to the clinical outcomes at TP3.
Results: There were no major adverse events. Only one patient dropped out because of repeated infusion reactions after infliximab read-ministration. At TP3, 41/42 patients (97.6%) had been reinfused because of a clinical relapse; one patient was in ongoing clinical remission without additional treatments.

ATI formation was seen in only in the one patient who dropped out of the study 18 weeks after retreatment due to repeated allergic reactions. No other safety concerns occurred during this long-term follow up after infliximab readministration. There were no correlations between ATI concentrations and outcome of clinical measures.

At TP3, a BASDAI 50% response was seen in 26/40 patients (65%), and partial remission was found in 13/41 (31.7%) patients, as compared to 22/42 (52%) patients and 17/42 patients (41.5%), respectively, at TP2 (both $p>0.05$).

The mean ($\pm$SD) BASDAI was 2.5 ± 1.8, 6.0 ± 1.4 and 2.6 ± 2.0, the median CRP (mg/l) was 1.3, 14.0 and 1.9 and the median ESR (mm/1h) was 8.0, 23.0 and 11.0 at TP1, TP2 and TP3, respectively. Changes were significant ($p<0.001$) between TP2 and TP3 but not between TP1 and TP3. Overall, the clinical response at TP3 was better in patients that were in partial remission TP2, compared to patients not in partial remission.
Conclusions: Discontinuation of long term infliximab therapy resulted in a clinical relapse in the majority of AS patients, while ongoing remission is a rare event. Readministration after discontinuation was generally safe and efficacious. The one patient with infusion reactions could have been predicted on the level of antibody formation.

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138 OUTCOME OF PATIENTS WITH ACTIVE ANKYLOSING SPONDYLITIS AFTER TWO YEARS OF THERAPY WITH ETANERCEPT: CLINICAL DATA SUPPORTED BY MAGNETIC RESONANCE IMAGING

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Background: Etanercept showed significant clinical efficacy in patients with active AS with no concomitant treatment with DMARDs and corticosteroids over 3 months.[1 2] Spinal inflammation, as assessed by MRI regressed after 6 and 24 weeks.

Here we report the clinical and MRI outcome after etanercept treatment over 102 weeks.
Methods: At baseline (BL) 30 patients were included in the study and received etanercept 2×25 mg/week s.c. for 12 weeks. Thereafter patients remained off therapy for a mean of 27 weeks. In case of a clinical relapse (defined as BASDAI and spinal pain ≥4 on a NRS), patients went into the open extension phase for another 2 years. Disease activity (BASDAI), function (BASFI) and mobility (BASMI) were assessed. MRIs were performed at BL, at 6, 24 and 102 weeks of etanercept therapy in the T1/Gd-DTPA and STIR sequences. The primary clinical outcome was a 50% improvement of BASDAI after 2 years, in comparison to the time point of etanercept readministration.
Results: Out of the 30 patients at BL, 26 (88%) were included in the open-label phase, 23 (89%) patients completed week 54, and 21 (81%) completed week 102. Both MRI sequences showed acute inflammatory lesions of the spine in all patients at BL.

In the ITT analysis after one year, 58% of the patients had achieved a BASDAI 50% response, 62% ASAS 40% response and 65% an 20% ASAS '5/6' response. After 2 years, a BASDAI 50% and an ASAS-40% response was still found in 54% of the patients.

Over 2 years of therapy the disease activity (BASDAI) improved constantly, with 2.7 ± 2.4 (BL:6.3 ± 1.6), while the mean CRP decreased from 15.3 ± 89 to 5.0 ± 8.1 mg/dl and the mean ESR from 22.8 ± 11.6 at BL to 9.0 ± 9.7 at week 102 (all $p<0.001$). Other clinical parameters improved similarly.

Spinal inflammation as assessed by MRI improved significantly already after 6 weeks, showed similar results after 24 weeks and was still decreased after 2 years of etanercept therapy.
Conclusions: This study confirms the persistent clinical efficacy and safety of etanercept over 2 years in patients with active AS. Spinal inflammation in MRI was reduced in all patients while on treatment with etanercept. However, inflammatory spinal lesions were not completely eradicated.

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139 INFLUENCE OF INFLIXIMAB THERAPY ON RADIOGRAPHIC PROGRESSION IN PATIENTS WITH ANKYLOSING SPONDYLITIS: RESULTS AFTER FOUR YEARS OF TREATMENT

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Background: Anti-TNF therapy with infliximab is clinically efficacious in patients with active ankylosing spondylitis (AS). This is consistent with improvement of spinal inflammation, as assessed by MRI. However, it is not yet clear whether structural radiographic changes are influenced.
Objective: To analyse the impact of infliximab therapy on radiographic progression of patients with AS after 4 years of treatment.
Methods: All 33 AS patients in this study are still participating in a still ongoing trial that has been reported[1 2] and were treated with infliximab for 4 years. Lateral radiographs of the cervical and lumbar spine were obtained at baseline (BL), after 2 years (FU1) and after 4 years (FU2) of follow-up and were scored blinded to patient identity using the mSASSS. Definite radiographic damage at BL was defined as at least one vertebral edge affected by a syndesmophyte (mSASSS$\geq$2 scoring points). Definite radiographic progression was evaluated by calculation of the percentage of patients with change from 0 or 1 to syndesmophytes or ankylosis (mSASSS>2). For comparison, any radiographic change (mSASSS>1) and radiographic change >the smallest detectable change (SDC),[3] recently determined at 2.9 in the mSASSS were calculated.
Results: The mean mSASSS was 11.6 ± 15.3 at BL, 12.5 ± 16.7 at FU1 ($p=0.005$) and 13.2 ± 16.7 at FU2 ($p=0.001$ and $p=0.02$ as compared to BL and FU1, respectively). This is less than the radiographic deterioration observed in the historical OASIS cohort.[4]

Definite radiographic progression was found in 7/33 patients (21.2%) between BL and FU1, in 5/33 (15.2%) patients between FU1 and FU2 and in 10/33 (30.3%) patients between BL and FU2.

Overall, only 4/33 patients (12.1%) with no radiographic damage at BL showed new chronic spinal lesions after 4 years.

Patients with radiographic damage at BL showed a significant worsening of chronic spinal changes at FU2 (1.7 ± 2.9, $p=0.005$ as compared to BL), in contrast to patients with no radiographic damage at BL ($p=0.001$ between groups).

There was no correlation between mSASSS scores and clinical parameters.
Conclusions: Some radiographic progression was observed after 2 and 4 years of infliximab therapy in active AS patients. However, the comparison to the historical OASIS cohort suggests that infliximab has some influence on the velocity and the degree of progression of structural changes in patients with AS. Larger studies are needed to finally prove that anti-TNF therapy inhibits structural damage.

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140 PROGRESSION OF RADIOGRAPHIC DAMAGE IN PATIENTS WITH ANKYLOSING SPONDYLITIS: ASSESSMENT OF DEFINITE CHANGE AND DEFINITION OF PREDICTIVE FACTORS

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Background: Patients with ankylosing spondylitis (AS) develop structural spinal changes associated with functional disability and handicap. Most characteristic are syndesmophytes and ankylosis, detected by conventional radiography. The quantification of such changes is crucial to assess the course of the disease and the response to therapy over time.
Objective: To analyse how radiographic progression in AS patients is best assessed and to study whether radiographic scores predict progression.
Methods: Overall, 116 AS patients with complete sets of lateral radiographs of the cervical and the lumbar spine at baseline (BL) and after 2 years of follow-up (FU) were randomly selected from 3 representative AS cohorts (two with anti-TNF-α (n = 58)[1 2] and one with conventional therapy (n = 58)). Structural spinal changes were assessed by the mSASSS[3] in concealed time order. 'Definite' change was defined as change from 0 or 1 to syndesmophytes or ankylosis (mSASSS$>$2). Syndesmophytes were differentiated from spondylophytes by setting a 45o angle as cut-off. The smallest detectable change (SDC) was calculated by using variance analysis as proposed recently.[4] For comparison, the proportions of patients with any change in the mSASSS, change of scores from suspicious to definite deterioration and also the smallest detectable difference (SDD) were calculated. Here we present preliminary results of the analyses; final results and conclusions will be presented during the EWRR meeting.
Results: The mean age of all 116 patients was 38.4$\pm$9.6 years (22–76 years), 68 patients (59%) were male and the mean disease duration was 11.0$\pm$8.2 years (1–37 years). The mean BASDAI was 5.1$\pm$2.2, the mean BASFI was 2.9$\pm$2.0 and the mean BASMI was 4.3$\pm$2.5. The mean CRP was 26.5$\pm$23.4 mg/l and the mean ESR was 27.8$\pm$20.3 mm/1h.

Radiographic damage at BL was detected in 88 patients (75.9%). At FU, any radiographic change (change$\geq$1 mSASSS unit) was seen in 49/116 patients (42.2%) and definite radiographic progression in 42/116 patients (36.2%).

The SDC was calculated at 2.9 and the SDD at 8.1 mSASSS units. Using the SDC, radiographic change was seen in only 24/116 patients (20.7%), while only 2/116 patients (1.7%) showed an mSASSS change$\geq$SDD at FU.

Of the 28/116 patients (24.1%) with no baseline damage, 5 patients (17.9%) showed new chronic lesions at FU.

Patients with definite radiographic damage at BL showed higher radiographic progression than patients without radiographic damage at BL (p = 0.002 between groups).
Conclusions: Radiographic progression in AS is well assessed by the mSASSS. This study confirms the validity of the score by assessment of definite change to syndesmophytes or ankylosis. The SDC and SDD are less sensitive and less useful. Baseline radiographic damage is predictive of future progression.

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141 FORMATTED ANTI-TNFα NANOBODIES SHOW SUPERIOR EFFICACY IN A COLLAGEN INDUCED ARTHRITIS MODEL IN MICE

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The advent of TNF blocking drugs has provided rheumatologists with an additional treatment option for management of patients suffering established rheumatoid arthritis. This new therapeutic strategy holds an enormous cost, which has made these drugs unavailable to many patients. Nanobodies, which are single-domain antigen binding proteins with high homology to human immunoglobulin VH domains, provide the opportunity to format highly potent drugs at lower costs than conventional antibodies. We aimed to evaluate the applicability of these Nanobodies as alternative TNF blocking agents in murine collagen-induced arthritis (CIA).

Nanobodies with neutralising activity for human TNF were isolated from immunised animals. These Nanobodies were formatted to assemble drugs with either bivalent TNF binding, or bivalent TNF binding with an added extra specificity for serum albumin. Albumin binding was previously shown to extend the half-life of small molecular drugs and it was suggested that a high turnover of serum albumin leads to an active targeting into inflamed tissues. Biological activity and pharmacokinetic properties of the drug candidates were tested in a TNF bioassay and by ELISA. Since no cross-reactivity with mouse TNF was observed, a mouse homolog construct was used to demonstrate efficacy in a mouse CIA model. Clinical scores were monitored and knee histopathology was examined at day 10 after start of treatment. The latter molecules were labeled with 99mTc and biodistribution was measured in vivo using gamma camera imaging.

Bivalent TNF binding, obtained after recombinant coupling of two monomeric anti-TNF Nanobodies, was found to dramatically enhance biological activity, which was determined to be markedly better than infliximab and adalimumab. We found that the formatted Nanobodies are easily expressed in *E coli* and are stable under various storage conditions. Including an albumin specificity substantially prolongs half-life, promotes accumulation in inflamed paws and improves significantly the therapeutic efficacy in a mouse model for RA. Clinical scores were significantly decreased, at least identical to the amelioration obtained by treatment with various conventional TNF blocking agents. Histopathological analysis of knee joints showed significantly reduced inflammatory infiltrate, cartilage destruction, proteoglycan depletion and bone erosion.

In conclusion, the present study demonstrates the suitability of Nanobody-based drugs as a flexible platform for development of cytokine blocking therapeutics. Due to the flexibility of the platform, highly potent bivalent drugs could be assembled and an additional specificity for serum albumin could be added to increase the half-life in these drug formats. Superior efficacy of these constructs was demonstrated in a mouse CIA model. Considering the low cost, tailored pharmacokinetic features and increased stability as compared to currently available TNF antagonists, we claim Nanobody-based drugs to be valuable alternatives for anti-TNF treatment and most likely other anti-cytokine treatments. Additional studies with anti-human TNF Nanobodies are currently ongoing.

142 SYSTEMIC DELIVERY OF AN ANTISENSE OLIGONUCLEOTIDE AND TREATMENT OF ESTABLISHED COLLAGEN-INDUCED ARTHRITIS BY A NOVEL CHARGE-REVERSIBLE LIPOSOMAL FORMULATION

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Although antisense oligonucleotide (ASO) and RNA interference technologies are highly promising for the specific silencing of genes, their clinical development has suffered from the lack of efficient drug delivery methods. In this study, we describe a novel method for the systemic delivery of ASOs to sites of chronic inflammation that is based on the use of a novel class of liposomes that are negatively charged under physiological conditions but become neutral and eventually positively charged during endocytosis when the pH drops. The negative charge allows their stable and aggregate-free travel of liposomes within the bloodstream, whereas the positive charge permits their fusion with the membrane and the escape of the cargo from the endosome. Once injected, these liposomes are distributed in the body in the same way as classic liposomes do but migrate to the tissues when there are openings in the capillaries such as those seen in inflammatory sites and tumours.

Here, we show that intravenous injection of a relatively low dose of charge-reversible liposomes carrying a CD40 ASO treats established collagen-induced arthritis in mice. It reduces all parameters of the disease measured that include clinical score, paw swelling and number of joints affected. Histologically, it reduces pannus formation and cartilage and bone damage. When compared to standard therapies, charge-reversible liposomes carrying CD40 ASO are more efficacious than anti-TNF and equally efficacious as dexamethasone in treating arthritis in this model. In the same conditions, alternative liposomal formulations have no effect on the above disease parameters.

Overall, charge-reversible liposomes offer significant advantages for delivering oligonucleotides when compared to current delivery methods and appear a very promising method for the in vivo delivery of oligonucleotides to sites of chronic inflammation.

143 AMELIORATION OF ARTHRITIS BY INTRA-ARTICULAR DOMINANT NEGATIVE IKKβ GENE THERAPY USING ADENO-ASSOCIATED VIRUS TYPE 5

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Nuclear factor (NF)-κB is highly activated in synovium of rheumatoid arthritis (RA) patients, and can induce transcription of many pro-inflammatory molecules. Phosphorylation of the inhibitor of κB (IkappaB) proteins is an important step in NF-κB activation and is under inflammatory conditions predominantly regulated by IκB kinase (IKK)β. Consequently, specific targeting of IKKβ locally in the joint using either small molecule inhibitors or gene therapy presents a sophisticated treatment option for arthritis. Adeno-associated virus serotype 5 (rAAV5) is currently considered the most promising vector for gene therapy in RA. In the present study we investigated the effect of inhibiting IKKβ in adjuvant arthritis (AA) in rats using AAV mediated intra-articular gene therapy. For this purpose rAAV5 containing the IKKβdn gene (AAV5.IKKβdn) or control rAAV5 encoding the enhanced green fluorescent protein (eGFP) gene (AAV5.eGFP) were injected into the right ankle joints either on day 6 or day 10 after adjuvant immunisation. The effect of intra-articular AAV5.IKKβdn gene therapy on paw swelling was measured by water displacement plethysmometry. In the rats treated with AAV5.IKKβdn in early arthritis (day 10) significantly reduced paw swelling was observed ($p<0.05$; AAV5.IKKβdn vs. AAV5.eGFP). Immunohistochemical analysis of synovial tissue revealed significantly reduced levels of IL-6 ($p=0.005$) and TNFalpha ($p=0.03$), whereas IL-10 levels were not affected. No significant effect was found on cartilage and bone destruction, or on matrix metalloproteinase (MMP)-3 and tissue inhibitor of MMPs (TIMP)-1 expression in the joints. Injection of AAV5.IKKβdn in the pre-clinical phase (day 6) only showed a marginal effect on the clinical course of arthritis and no significant changes in the cytokine profile were detected. Interestingly, MMP3 expression was decreased after AAV5.IKKβdn injection on day 6, suggesting a role for IKKβ in the expression of MMP3 early in the disease. In addition to the animal studies, we also demonstrate that our vector is capable of transducing human whole synovial tissue biopsies ex vivo, resulting in reduced IL-6 production after TNFα stimulation ($p=0.03$).

In conclusion, we demonstrate for the first time that rAAV5 can be used to successfully target an intracellular signaling intermediate like IKKβ in the synovium, resulting in reduced severity of inflammation in AA in vivo and pro-inflammatory cytokine production in human RA synovial tissue ex vivo.

144 EFFICIENT NEW CATIONIC LIPOSOME FORMULATION FOR SYSTEMIC DELIVERY OF SMALL INTERFERING RNA SILENCING TNF-α IN EXPERIMENTAL ARTHRITIS

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Objective: TNF-α is the prominent cytokine in rheumatoid arthritis (RA), secreted mainly by macrophages. A direct method for restoring the immunological balance in RA is the use of small interfering RNA (siRNA) for silencing the TNF-α transcript. This study aimed at determining the therapeutic effect of systemic administration of TNF-α siRNA in an experimental model of RA, and optimising its delivery using new liposome formulations.
Methods: Mouse macrophages were transfected with siRNA targeting TNF-α and expression was measured by quantitative PCR and ELISA. Therapeutical effect was assessed in collagen-induced arthritis after intravenous delivery of TNF-α siRNA. The delivery was optimised using a DNA carrier for complexation with the cationic liposome RPR209120/ DOPE. The TNF-α and other cytokines were measured in sera and joint conditioned media. Clinical course of the disease was monitored by paw thickness measuring radiological and histological scoring.The biodistribution was determined using a fluorescent siRNA.
Results: In vitro, the TNF-α siRNA efficiently and specifically modulate the TNF-α expression in macrophages at both mRNA and protein levels. In vivo, complete cure of collagen-induced arthritis was observed when the TNF-α siRNA was delivered weekly, complexed with the liposome combined to a DNA carrier. A 50–70% inhibition of the articular and systemic TNF-α secretion was detected in the siRNA-injected groups, correlated with an important decrease of IL-6 and MCP-1 levels. Main targeted organs by siRNA were liver and spleen, the addition of the RPR290120 liposome and carrier DNA significantly increased the organs uptake.
Conclusion: We demonstrate the efficiency of a systemic delivery of a siRNA silencing TNF-α in CIA by using a liposome-carrier system as a way to face the methodological limitations in vivo.

145 VOLUMETRIC COLOUR CODED SHAPE MAPPING: A NOVEL DYNAMIC CONTRAST ENHANCED MRI IMAGING TECHNIQUE

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Background: Dynamic contrast enhanced (DCE) MRI consists of a series of fast scans acquired during injection of a Gadolineum (Gd) containing contrast agent. Depending on the amount and structure of local microvascularisation different enhancement patterns will be recorded. The enhancement patterns can be described by different parameters such as maximal enhancement, time to enhancement, initial slope of increase and shape pattern. It has been suggested that these shape patterns may relate to disease severity. Arthritis is known for its inhomogeneous degree and distribution of synovial inflammation within the joint. Using only one region of interest to evaluate synovitis might lead to sampling error resulting in over- or underestimation of synovitis.
Objective: To present a novel 3-dimensional (3D) volumetric analysis and display method with millimeter resolution for DCE-MRI by generating colour coded shape maps. Furthermore, to visualise differences in enhancement patterns emphasising heterogeneity within synovial tissue.
Methods: The DCE-MRI protocol for the knee joint was performed with a 1.5 Tesla scanner (GE Signa) and consisted of 20 subsequent 3D-Spoiled GRE sequences. Together with the maps of Maximum Enhancement (ME), Time to Enhancement, and Initial Slope of Increase obtained from time enhancement curves, we have developed software to categorise 5 characteristic curve shapes (I-II-III-IV-V) as described previously (van Rijswijk *et al. Radiology* 2004;493), and displayed them in maps. We created a colour coded system that visualises these characteristic curve shapes in the whole 3D (multislice) imaged joint area. All maps were analysed for occurrence and distribution of different shape curves and ME-values.
Results: Our study group consisted of 14 early arthritis (<1 year's duration) patients, of which 7 (probable) RA and 7 non-RA. No significant differences in clinical parameters between both groups were seen. ME maps showed a consistent increased enhancement in areas of synovial inflammation. In each patient all different curve types were seen, although to different extents. The colour-coded shape maps revealed a striking heterogeneous distribution of different curve types throughout the joint, and even within a ROI with a homogeneous maximal enhancement different curve types were seen.
Conclusion: Colour coded shape maps in DCE-MRI in a 3D volume clearly show the heterogeneity within the affected synovial tissue even within areas with a homogeneous maximal enhancement. This emphasised that only ME maps to identify the uptake pattern are not accurate enough to evaluate synovitis and a region of interest approach might lead to sampling error.

146 BOSENTAN REDUCES THE NUMBER OF NEW DIGITAL ULCERS IN PATIENTS WITH SYSTEMIC SCLEROSIS

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Objective: The aetiology of digital tip ulcers (DTU) in systemic sclerosis (SSc) is unclear, but endothelin is implicated. We report a randomised clinical trial (RCT) with bosentan, a dual endothelin receptor antagonist, in which we sought to confirm the reduction in number of new digital ulcers (DTU) observed in a previous trial in patients and to evaluate potential effects on healing.
Methods: Study eligibility included a diagnosis of SSc and at least one recent active DTU. In subjects with more than one active DTU, a ''cardinal ulcer'' was identified based on location, clinical impact and amenability to healing. Subjects received bosentan at 62.5 mg bid for 4 weeks then 125 mg bid for 20 to 32 weeks or placebo (PBO) in 1:1 blinded randomisation. All DTUs were assessed at 4 weekly intervals. Co-primary endpoints were time to complete healing of the cardinal

ulcer and the number of new DTUs that developed during 24 weeks of study. Secondary endpoints included SHAQ-DI, safety and tolerability.
Results: The 188 subjects studied were well balanced between bosentan and PBO by age, gender, SSc classification, smoking history and concomitant medications. Total number of new ulcers per patient up to 24 weeks was 1.9 + 0.2 on bosentan versus. 2.7 + 0.3 on PBO ($p=0.035$, Pitman permutation). The reduction was more pronounced in patients with more than 3 active digital ulcers at baseline. The effect on healing was comparable between bosentan and PBO, DTUs persisting in 50% of all patients for up to 24 weeks. Bosentan therapy was associated with improved hand function. SHAQ ''dressing'' improvement was significant for bosentan over PBO at 24 weeks ($p=0.033$) and trended in favour of drug for ''eating'' at 24 weeks ($p= 0.098$). SHAQ Visual Analogue Scales improved on bosentan for pain at 12 weeks ($p=0.034$). SAEs were uncommon. ALT/AST > 3X ULN was more frequent on bosentan (10.5%) than PBO (1.1%).
Conclusions: This second large RCT confirms that bosentan reduces the number of new DTUs in patients with SSc and that this effect is associated with reduced pain and improved hand function. Bosentan therapy does not appear to facilitate healing of active DTUs.

147 THE EFFECTS OF BLOCKING C5A IN PATIENTS WITH ACTIVE RHEUMATOID ARTHRITIS

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Introduction: All complement pathways eventually lead to formation of anaphylotoxin C5a, which is believed to contribute to the activation and influx of C5a-receptor (C5aR) positive cells, such as monocytes and neutrophils, into the joints of patients with rheumatoid arthritis (RA). Studies in animal models for arthritis have suggested therapeutic potential of C5aR blockade. In this double-blind, placebo-controlled proof-of-principle study, the effects of an oral C5aR antagonist in RA patients were evaluated.
Methods: 21 patients with active RA were randomised to 28 days of active treatment with the C5aR antagonist AcF-(OpdChaWR) (PMX53) versus placebo in a 2:1 ratio. Serum concentrations of PMX53 were determined for pharmacokinetic analysis. For pharmacodynamic analysis, synovial biopsy specimens were obtained by needle arthroscopy at baseline and day 29. Using immunohistochemistry and computerised digital analysis, we evaluated cell infiltration and cytokine expression in the synovium. In addition, DAS28 and ACR20 responses were assessed. Statistical analysis was performed using t-tests.
Results: All patients completed the study; no serious adverse events occurred. The area under the curve (AUC) varied from 1.8 to 130.7 nmol hr/l, mean 40.8 nmol hr/l. DAS28 and ACR20 showed no significant clinical improvement in the group treated with PMX53 compared to placebo. There was no correlation between the AUC and the clinical response according to the DAS28. No decrease in cellular infiltration, including key biomarkers associated with clinical efficacy, of the synovium was observed in either groups.
Conclusion: C5aR blockade does not result in a reduction of synovial inflammation or clinical improvement, in spite of the fact that PMX53 serum levels are reached that lead to C5aR-mediated cell activation blockade in vitro. These data suggest that C5a does not play a pivital role in synovial inflammation in RA patients.

148 ALLOREACTIVE NK CELLS IN THE CONTROL OF SYSTEMIC AUTOIMMUNE DISEASE AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION

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Introduction: Bone marrow transplantation (BMT) is an effective treatment for several systemic autoimmune diseases (AID), including collagen-induced arthritis (CIA). Here, we analysed the contribution of alloreactive NK cells from the donor in the treatment of arthritis following allogeneic BMT.
Methods: CIA was used as a model for chronic B cell-mediated arthritis. Arthritic mice were subsequently subjected to sublethal total body irradiation of 6.0 Gy and anti-CD40L mAb before transfer of total bone marrow (BM) and/or in vitro cultured donor NK cells from C57BL/6 or F1 mice. Pathogenic autoantibodies, donor chimerism as well as antigen-specific host B cells were measured by ELISA and FACS, respectively. Anti-NK1.1 mAb was used to deplete donor NK cells after BMT.
Results: The induction of stable, long-term and multi-lineage donor chimerism could be observed after allogeneic BMT. No graft-vs-host disease (GVHD; defined by weight loss and histological abnormalities of liver, gut and skin) was observed. Allogeneic BMT not only arrested disease progression, but also significantly suppressed the production of pathogenic autoantibodies This was associated with the elimination of antigen-specific host B cells. By using allogeneic BM cells from F1 mice, we showed that NK cells, but not T cells, were responsible for these effects. Moreover, depletion of donor NK cells resulted in less effective treatment, while transfer of in vitro cultured NK cells increased the elimination of host immune cells, including antigen-specific B cells.
Conclusion: The beneficial effects of allogeneic BMT were most likely caused by the elimination of host B cells producing pathogenic autoantibodies as these disappeared rapidly after BMT. Alloreactive NK cells from the donor are involved in this process. These results provide a rationale for the use of donor NK cells in treating systemic B cell-mediated AID like chronic arthritis.

149 ANTILYMPHOCYTE GLOBULINS TRIGGER APOPTOSIS IN CD4+CD28 T-CELLS BY FAS INDEPENDENT MECHANISMS

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Background: Polyclonal antilymphocyte globulins (ATG) are directed against Jurkat-cells and currently used for treatment of severe aplastic anaemia, organ allograft acute rejection and graft-versus-host disease. ATG treatment results in major depletion of peripheral blood lymphocytes, which contributes to its overall immunosuppressive effects. Its precise mechanism of action, however, is unclear.

In various chronic inflammatory diseases, including rheumatoid arthritis, ankylosing spondylitis, Wegener's granulomatosis and multiple sclerosis, the occurrence of pro-inflammatory, cytotoxic CD4+CD28- T-cells has been considered as markers of premature immunosenescence. The clonality and longevity of CD4+CD28- T-cells is associated with defects in apoptotic pathways and thus explain the persistence of these cells over years.
Objectives: To study the in vitro effects of ATG on apoptosis, activation and cytokine production of CD4+ T-cell subsets and to examine the in vivo effects of ATG on peripheral levels of CD3+CD4+CD28- T-cells.
Material and Methods: Cells from short-term cell lines were incubated with polyclonal rabbit ATG (Fresenius) and three colour FACS analyses were performed for CD4, CD28, CD3, CD25 and AnnexinV as well as for intracellular production of IFN-γ, TNF-α and IL-4.

Then 5 patients receiving ATG (38.6 ± 11.8 years old) and 11 patients without ATG treatment (50.5 ± 6.7 years old) after organ transplantation were randomly enrolled in a prospective design. Percentages of CD28- out of the CD3+CD4+ peripheral blood mononuclear cells were determined before and 6 hours after ATG application by FACS analysis. Laboratory investigators were blinded for ATG treatment of patients.
Results: In vitro 100 μg/ml ATG for 18 hours induced apoptosis in both CD4+CD28+ and CD4+CD28- T-cells (72.8 ± 17.8 and 87.5 ± 10.3 AnnexinV+ cells, respectively) in comparison to unspecific rabbit IgG as negative control (14.0 ± 3.5 and 4.4 ± 2.4 AnnexinV+ cells, respectively) (both with $P=0.018$). This effect was not inhibited by the blocking anti-Fas antibody ZB4. ATG at 100 μg/ml also induced the expression of CD25 about four fold on CD4+CD28+ and 12-fold on CD4+CD28- T-cells compared to rabbit IgG (both with $P=0.018$). Incubation of cells with ATG at dosages of 1000 μg/ml for 4 hours stimulated the production of IFN-γ and TNF-α but not of IL-4 in both CD4+ T-cell subsets.

In vivo data showed that fresh circulating CD3+CD4+CD28- T-cells decreased from 3.7 ± 7.1 to $0\pm0\%$ ($P=0.043$) in ATG treated patients but not in control patients with 2.9 ± 2.9 and $2.7\pm2.9\%$.
Conclusions: In vitro, ATG induces activation with CD25 expression and apoptosis in both CD4+CD28+ and CD4+CD28- T-cells. These data could be confirmed in vivo with percentages of CD3+CD4+CD28- T-cells being reduced after treatment with ATG.

150 ANALYSIS OF APOPTOSIS VERY EARLY AFTER INITITATION OF INFLIXIMAB TREATMENT IN RHEUMATOID ARTHRITIS PATIENTS

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Objective: Previous work has shown that there is a marked decrease in synovial inflammation 48 hours after initiation of infliximab treatment, which could not be correlated to apoptosis induction at that time point. It

has been suggested that apoptosis induction might occur very early after the first infusion. Therefore, we analysed whether treatment with infliximab would lead to apoptosis induction within one hour after treatment.

Methods: Five rheumatoid arthritis (RA) patients with a mean Disease Activity Score (DAS 28) of 5.1> received a first infusion of infliximab 3 mg/kg intravenously in addition to stable methotrexate therapy. All patients were subjected to serial synovial biopsies obtained by arthroscopy of the knee joint before, and 1 hour after completing the first infliximab infusion. Cellular infiltrate was characterized by immunohistochemical analysis. Apoptosis of synovial cells was examined by the presence of terminal deoxy (d)-UTP nick end labelling (+) cells (TUNEL (+) cells) and expression of caspase-3 by immunohistochemistry. Tonsillar tissue was used as a positive control. We confirmed nuclear blebbing by electron microscopy (EM) as a gold standard for apoptosis detection.

Results: One hour after infusion of infliximab the number of CD68+ sublining macrophages, CD3+ T cells, CD22+ B cells and CD38+ plasma cells was on average unaltered. In 4 of 5 patients there was no increase in TUNEL + cells. In only one patient we observed an increase in TUNEL+ cells from 0 cells/mm2 to 26 cells/mm2 after treatment. Although the highest number of caspase-3 + cells, 16 cells/mm2, was detected in this patient before treatment, there was no increase after infliximab treatment. Electron microscopy showed no apoptotic lymphocytes or macrophages after treatment. The patient with a slight increase in TUNEL + cells was a good responder according to the EULAR response criteria at 16 weeks. From the other 4 patients 2 had a good EULAR response, 1 a moderate response and 1 was non-responder at 16 weeks.

Conclusions: There was no clear cut increase in apoptotic cells 1 hour after completion of the first infliximab infusion. The results presented here do not support the notion that the marked decrease in synovial inflammation that can be observed within the first two days after initiation of infliximab can be explained by induction of apoptosis in the synovial compartment. Whether apoptosis induction plays a role in the interval between 1 hour and 48 hours remains to be shown.

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151 INVESTIGATION OF THE ROLE OF P38 MAPK ISOFORMS IN THE RHEUMATOID SYNOVIUM

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Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown aetiology causing damage to articular structures, including cartilage and bone, with progressive disability. Thickening of the synovial membrane with proliferation of fibroblast-like and macrophage-like synoviocytes is observed in affected joints as well as extensive synovial infiltration of inflammatory cells, including B and T lymphocytes, macrophages and dendritic cells.

The p38 mitogen/stress-activated protein kinase (MAPK/SAPK) signalling pathway constitutes one arm of the MAPK cascade, involving a complex series of signalling pathways, which can be activated by multiple stimuli including TNF, toll-like receptor activation and environmental stress. Cellular response to these stimuli can be cell type and stimulus specific. There are four p38 MAPK isoforms: α, β, γ and δ of which α, β and δ are found in varying levels in inflammatory cells. p38 MAPK is thought to play a role in the pathogenesis of RA although most research and therapy has so far been focused on p38α and little is known of the relative contribution of the other isoforms to disease progression.

The aim of the study was to correlate p38 isoform expression in the rheumatoid synovium with the visual analogue score (VAS) done at arthroscopy as a measure of synovitis. Synovial biopsies taken from patients diagnosed with RA by ACR criteria were snap frozen in liquid nitrogen and 5 µm sections cut. Immunohistochemistry was then performed using antibodies against specific p38 isoforms and phospho (activated) p38. Semi-quantitative analysis was used to score positive staining of the lining and sublining layers of the synovium and correlated with the VAS scores. Spearman's rank correlation coefficient was used to analyse the data.

A strongly positive correlation between increased VAS score and reduced expression of p38δ in the sublining layer ($p=0.008$) was found, whereas p38α expression showed no correlation ($p=0.8$) in the 8 biopsies studied. These data indicate that specific p38 MAPK isoforms expressed in the synovial sublining layers may correlate with the degree of inflammation and suggest individual roles for different p38 isoforms in disease progression in RA.

152 INFLUENZA VACCINATION AS MODEL FOR TESTING IMMUNE MODULATION OF ANTI-TNF AND METHOTREXATE THERAPY IN RHEUMATOID ARTHRITIS PATIENTS

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Background: We have reported that the in vivo immune response after polysaccharide antigen stimulation (pneumococcal vaccination) in rheumatoid arthritis (RA) patients is decreased in methotrexate but not in anti-TNF treated patients (Rheumatology 2005 Nov 15 [Epub]). We now wanted to investigate how a polypeptide antigen (influenza vaccination) performs in the same patients.

Objective: To compare serological response to influenza vaccine in patients with long-standing rheumatoid arthritis (RA) treated with TNF-blockers and/or methotrexate and healthy controls

Patients and Methods: Altogether 149 patients with established RA and 18 healthy subjects among medical staff were vaccinated. 50 patients were treated with TNF-blockers (etanercept or infliximab) in combination with methotrexate (TNF-blockers+MTX), while 62 patients were treated with TNF-blockers alone or with other DMARDs (TNF-blockers without MTX). 37 patients were treated with methotrexate without TNF-blockers (MTX). Controls consisted of 18 hospital staff.

Vaccination was performed during the winter seasons 2000/01 and 2001/02 with commercially available vaccine (Influvax and Vaxigrip) both containing 15 µg HI of each of 2 A strains (H1N1 IVR-116 and H3N2 ResVir-17) and 2 B strains (B1 B/Yamanashi/116/'98 and B2 B/Guangdong/120/'00). Serum samples were collected prior to and 4–6 weeks after vaccination and titrated against all 4 strains using haemagglutination inhibition assay (HIA). A positive immune response was defined as a fourfold or higher increase compared to pre-vaccination titre levels. A titre ⩾40 was considered protective.

Statistical analyses were performed using Mann-Whitney U-test. Because of differences at baseline we used a logistic regression model to adjust for age, gender and thereafter disease duration and prednisolone dosage one at a time.

Results: Post-vaccination titre levels increased significantly in all groups compared to pre-vaccination levels (table 1). A positive immune response to combinations of strains (H1N1+H3N2=A strains,

Abstract 152 Table 1 Percent of patients with pre-vaccinations titres <40 and positive immunization response (⩾ 4 fold titre increase) to different strains

Strains	TNF-blockers without MTX n=62	TNF-blockers+MTX n=50	MTX n=37	Controls n=18
H1N1	73.7% (42/57)	68.1% (32/47)	93.9% (31/33)	69.2% (9/13)
H3N2	77.1% (37/48)	57.5% (23/40)	81.3% (26/32)	38.5% (5/13)
B1	86.4% (38/44)	77.4% (24/31)	100% (23/23)	71.4% (5/7)
B2	81.6% (40/49)	64.7% (22/34)	91.3% (21/23)	75.0% (6/8)

Abstract 152 Table 2 Odds ratios, 95% confidence intervals (95% CI) and p-values after adjustment for age, gender and thereafter for disease duration and prednisolone dosage

	TNF-blockers without MTX vs MTX			TNF-blockers+MTX vs MTX		
Strains	p	ODDS	95% CI	p	ODDS	95% CI
A	**p=0.027**	0.339	0.130–0.886	**p=0.011**	0.262	0.094–0.733
B	p=0.134	0.466	0.171–1.266	**p=0.011**	0.246	0.084–0.723
A+B	**p=0.003**	0.223	0.084–0.595	**p=0.002**	0.184	0.064–0.531

B1+B2=B strains or A+B strains) was significantly better for the MTX group also after adjustments in the regression model (table 2). Individuals with protective levels before vaccination responded less well to vaccination as a group. The substantial number of individuals in the controls with protective pre-vaccination levels was the reason for excluding this group from further analysis.

Conclusions: The in vivo immune reactions to polypeptide stimulation were clearly different when compared to polysaccharide stimulation (pneumococcal vaccination). RA patients treated with MTX without TNF blockers had significantly better serological response to influenza vaccination compared to those receiving TNF blockers alone or in combination with methotrexate and/or other DMARDs. However, the immune response is sufficiently large as to warrant influenza vaccination to all RA patients regardless of treatment. Age, gender, disease duration and prednisolone dosage did not significantly influence the immune response. High pre-vaccination protective levels in controls were probably explained by previous free influenza vaccination offered by the hospital.

153 TUMOUR NECROSIS FACTOR ALPHA RECEPTOR GENE TRANSFER IN A MURINE MODEL OF SJÖGREN'S SYNDROME HAS DISEASE MODIFYING EFFECTS

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Sjögren's Syndrome (SS), a chronic autoimmune disease is characterised by focal mononuclear infiltrates (mainly CD4+ cells) in the salivary glands (SGs) and decreased saliva production. Current therapies for SS are unsatisfactory. A disappointing success rate of systemic treatment with TNFα-blocking drugs in SS patients compared to rheumatoid arthritis (RA) patients is recently reported. This could be due to the lack of sufficient levels of the TNFα-blocking drugs at the active sites of disease such as the salivary glands. We hypothesised that although systemic TNFα-blockade in SS patients had moderate disease modifying effects, local salivary gland TNF receptor gene transfer, mediated by a recombinant adeno-associated viral (rAAV) vector, would decrease lymphocytic infiltration and increase salivary flow in non-obese diabetic (NOD) mice. NOD mice, a model of SS, exhibit focal lymphocytic infiltrates and a characteristic age and gender (female) related decline of salivary flow. Vectors, were delivered locally, via cannulated ducts with retrograde infusion to the SGs. Mice received rAAVTNFR, as active treatment (T). Control groups received either saline (S), rAAV, encoding B-galactosidase (rAAVLacZ) (C-) or rAAVhIL10 (C+, Kok *et al*, 2003). Mice received vector at age 8 wk, before onset of sialadenitis and were sacrificed at week 16. Blood glucose levels were monitored weekly and mice with >400 mg/dl were treated with insulin by injection (q24 h) to limit diabetes related dehydration. Saliva collection and focus scores were performed, as previously described (Yamano *et al*, 1999). Mice receiving rAAVTNFR or rAAVhIL10, had a salivary flow rate increase at week 16 compared to week 8 of (l/20min; mean ± SEM) 46±16 (T) and 37±14 (C+). Mice receiving either saline or rAAVLacZ had a decline in salivary flow of −20±14 (S) and −2±10 (l/20min) (C−). There was a significant difference between C− vs. T ($p=0.028$) and C− vs. C+ ($p=0.045$). There was no significant difference between T and C+ or C− and S. Mice receiving vectors, had focus scores at 16 wk of (mean ± SEM) 2.02±0.16 (S), 2.14±0.18 (C−), 1.17±0.24 (T) and 1.22±0.22 (C+) with a significant difference between C− and T ($p<0.001$). Similarly, for mice receiving C+ vs. C− there was a significant difference ($p=0.005$). We did not observe any significant differences between C+ and T nor between C− and S. Local rAAV mediated TNF receptor gene transfer modifies sialadenitis in NOD mice resulting in decreased tissue inflammation and increased saliva production. These findings suggest, in contrast to systemic administration of a TNFα-blocking agent, that local gene therapy in SGs using rAAVTNFR may be beneficial for SS patients.