Molecular Regulation of Gene Expression in Chondrocytes by Inflammatory Mediators

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Message from World Health Organization

Health needs assessment of musculoskeletal conditions: an international bone and joint burden of disease study

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Introduction The Bone and Joint Monitor Project was developed to quantify the global burden of musculoskeletal conditions and develop strategies for their prevention. Experts within the Monitor Project have worked previously with officers at the World Health Organization (WHO) to estimate morbidity and mortality associated with rheumatic conditions. The present collaboration seeks means of providing additional and more current burden data.
Objective To develop recommendations for performing epidemiological studies in sample populations with musculoskeletal conditions and problems, accounting for determinants and consequences to the individual and society.
Methods Recommendations have been developed identifying the most relevant domains for measuring and monitoring the various musculoskeletal conditions by review of epidemiological data on occurrence, determinants and outcomes, and by expert opinion. Instruments that measure these domains were reviewed.
Results The domains recommended follow the principles of the WHO International Classification of Functioning, Disability and Health [1,2], and consider: health condition; body function and structure; activity limitation; participation restriction; personal and environmental contextual factors; and, in addition, the resource utilisation and social consequences. The musculoskeletal conditions and problems considered were osteoarthritis, inflammatory arthritis, osteoporosis, spinal problems, musculoskeletal trauma and injuries, and musculoskeletal pain with restricted activity. The selection of indicators for each domain considered the feasibility of their use in a health interview survey (HIS), a health examination survey (HES), a register or a clinical study. Consensus on case definition was reached depending on the study methodology. For example, osteoporosis defined by bone densitometry cannot be ascertained in an HIS, whereas the outcome of osteoporosis (i.e. fragility fracture) can be. Osteoarthitis can be identified as joint pain in an HIS but the preferred definition is pain with X-ray changes and can only be ascertained in an HES. Previously validated generic and disease-specific instruments have been identified that include indicators for all or most of the recommended domains for the consequences of the different conditions and problems. The indicators of the domains for resource utilisation and social consequences and feasibility for collection will vary in different socioeconomic and geographic areas. Guidance on sampling methods is also being developed.
Conclusions The comparability of data collected across the globe will improve by the application of agreed upon indicators that consider key domains for the different musculoskeletal conditions and problems in epidemiological studies conducted in different populations.
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ORAL PRESENTATIONS

Session I – Genomic/genetic/proteomic: the added value to arthritis research and treatment

1
Osteoarthritis: degeneration of matrix or cells?

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Osteoarthritis is the most common disabling condition of man in the Western world. Biologically, osteoarthritis implies the (symptomatic) degeneration of the whole joint with many structures involved such as subchondral bone and the periarticular soft tissues. Still, the degeneration of the articular cartilage appears to be the focus of the structural changes taking place during the osteoarthritic disease process.
Cartilage degeneration implies mostly a failure of the extracellular matrix of the articular cartilage. The matrix represents the functional component responsible for the biomechanical integrity of the tissue. Thus, many studies over decades in osteoarthritis and cartilage research have focused on the understanding of degenerative or degradative processes taking place within the matrix. This has led to significant continuous advances in our understanding of the biochemistry and pathobiochemistry of molecules and their assembly within the cartilage matrix.
The cells of the tissue, which do not exert per se structural functions within the tissue, were considered largely responsible for the proper matrix turnover. They were thought to react to external mediators and biomechanical stimuli or assaults and to be more or less able to compensate for continuous (bio)mechanical tissue destruction. More recently, however, besides matrix (patho)biochemistry, the cellular phenotype during the disease process has come more and more into the focus of interest. This was induced on the one hand by upcoming (mol-

ecular) technology, but also by increasing evidence that also the cells degenerate per se. Osteoarthritic chondrocytes are not only no longer able to compensate for matrix degeneration, but themselves are (co)initiators and (co)promoters of the disease process.

2
Genetics of osteoarthritis

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Epidemiological studies have demonstrated a major genetic component to osteoarthritis (OA), with heritability estimates of over 50% for most joint sites. These studies have also highlighted differences in the degree of OA heritability between joint sites and between the sexes, implying a high level of heterogeneity.

We published a genome-wide scan in 1999. We had focused on families containing siblings with severe large-joint OA ascertained by joint-replacement surgery. Our data showed that OA genetic susceptibility did exhibit joint specificity and that this susceptibility had a greater role in female disease. During the past 5 years we have been investigating our linkage regions and we have so far identified *FRZB* (chromosome 2q32.1), *COL9A1* (6q12-q13), *BMP5* (6p12.1) and *IL4R* (16p12.1-p11.2) as encoding for OA susceptibility. Common variants at these genes affect either the structural properties of the protein (*FRZB* and *IL4R*) or the transcription of the gene (*BMP5* and *COL9A1*). The variants are particularly relevant to the development of hip OA in females.

What is particularly interesting about our recent discoveries is that the proteins encoded for by *IL4R*, *BMP5* and *FRZB* are involved in chondrocyte cell signalling and signal transduction pathways. It appears probable, therefore, that OA genetic risk for the hip is principally accounted for by aberrant cell signalling. This was not anticipated.

In this presentation I will focus on our latest genetic findings. I will also discuss the results from other studies. A number of OA genome-wide scans have been performed, some on large joints and others investigating hand disease. Many loci appear unique to one particular study, with only some loci being positive in multiple studies. The reasons for this will be discussed.

A concern often expressed at orthopaedic and rheumatology meetings is that genetic linkages and associations are not consistently reproduced. These are reasonable criticisms but it needs to be remembered that the genetic component of a complex trait such as OA will not be mediated by fully penetrant risk alleles. Instead, any one allele will contribute only a fraction of the overall risk and this allele will, by chance, have varying frequencies in different cohorts. It is unreasonable, therefore, to expect a linkage or an association in one cohort to be replicated in all cohorts.

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3
Genetic control of T-cell autoimmunity and arthritis

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Inbred animals are useful for studies of the identification of genes associated with rheumatoid arthritis (RA) since they are a more efficient tool for identification genes controlling complex diseases. There are several arthritis models, each of which may reflect various variants of the heterogeneity of RA in humans. Examples are collagen-induced arthritis and pristane-induced arthritis, which both fulfil the clinical diagnostic criteria for RA.

Type II collagen (CII) is immunogenic and contains peptides that can be bound to major histocompatibility complex (MHC) class II and presented to T cells, whereas pristane is not immunogenic by itself. Both diseases are genetically complex and the susceptibility is, as RA, dependent on many polymorphic genes operating in concert. So far two genes in this concert have been identified; the MHC class II Ab gene in the mouse [1] and the Ncf1 gene in the rat [2]. The Ncf1 protein is a part of the NADPH oxidase complex involved in generation of the inducible oxidative burst. The discovery of the Ncf1 polymorphism led to a new proposed pathway in which oxygen radicals modify antigen presentation and the resulting activation of autoreactive T cells. This hypothesis has now been further documented by the identification of an Ncf1 mutation in the mouse that reproduces the effects earlier observed in the rat. Mice with the deficient Ncf1 allele, and expressing the MHC class II allele Aq, binding CII peptides, could be shown to be dramatically more susceptible to collagen-induced arthritis, and also developed a chronic form of arthritis. Interestingly, the immune response to CII was enhanced by the Ncf1 deficiency linking the Ncf1 pathway to the adaptive immune response.

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4
Genome-wide single nucleotide polymorphism analyses on rheumatoid arthritis

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Although the etiology of rheumatoid arthritis (RA) is still unknown, it is generally believed that a combination of multiple polymorphic genetic factors and environmental contributions lead to the pathogenic autoimmune reactions characterized by destructive polyarthritis and various extra-articular inflammatory involvements. A genetic approach to identify RA-associated genes from the whole genome is proposed to be one of the promising methodologies to elucidate the etiology of RA. Although multiple genome-wide linkage studies and many association studies to test an individual gene that is functionally relevant to RA have been performed to identify RA-associated nonhuman leukocyte antigen genes, no region or gene has been conclusively shown to be one of the RA-susceptible loci or genes. Therefore, a linkage disequilibrium approach using single nucleotide polymorphisms (SNPs) as genetic markers to identify disease-associated genes was considered a promising alternative method for RA, as well as other common diseases with complex genetic predispositions.

Our RIKEN SNP Research Center has established a high-throughput SNP-genotyping system and has been investigating multiple common diseases using a whole-genome gene-based linkage disequilibrium mapping approach. In 2003, we reported that functional variants of the PADI4 gene were associated with RA. PADI4 is an enzyme that converts arginine residue in proteins to citrulline. The citrullinated proteins are targets of the most specific autoantibodies in RA. We have identified this gene in a whole-genome, hypothesis-free approach. Therefore, this made our methodology strongly reliable in that the RA-associated gene identified by our hypothesis-free approach was functionally strongly relevant to the pathogenesis of RA.

Recently we also found that SLC22A4 and RUNX1 are associated with RA. SLC22A4 is an organic cation transporter with unknown physiologic function, and RUNX1 is a hematologic transcriptional regulator that has been shown to be responsible for acute myelogenic leukemia. It is suggested that the association of RUNX1 with RA is due to its regulation of expression of SLC22A4. Because the physiologic function of SLC22A4 is still unclear, further investigation is needed into how SLC22A4 affects RA susceptibility. Although the association of RUNX1 with RA was identified as a regulatory factor of SLC22A4, it is possible that RUNX1 is a key molecule in autoimmunity, as it has been reported to be associated with systemic lupus erythematosus and psoriasis, two other autoimmune diseases.

5

Rheumatoid arthritis: a heterogeneous disease evaluated by DNA-microarray technology

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The molecular pathogenesis of rheumatoid arthritis (RA) is still poorly understood and its clinical course can vary widely. We apply a systems biology approach to gain insight into the complex pathogenesis and disease heterogeneity. Genomics studies revealed considerable heterogeneity in global gene expression signatures between synovial tissue specimens from different patients with RA. Based on the molecular signatures, at least two distinct subsets of RA tissues could be identified. One class revealed abundant expression of gene clusters indicative of an ongoing activation of the adaptive immune response, whereas the other class resembled the expression pattern of osteoarthritic tissues, which are characterized by a low inflammatory gene expression signature and increased tissue remodeling. The molecular heterogeneity is featured not only at the whole synovial tissue level, but also at the level of fibroblast-like synoviocytes (FLS) cultured from those tissues. One of the most impressive features of our gene expression profiling studies is the clear correlation of the FLS phenotype with that of paired synovial tissue from which the cells were derived. One class of FLS is tightly related to the presence of lymphocytes in the lesions, whereas the other class of FLS suggests that synoviocyte-mediated invasion appears to be less dependent on infiltrating immune cells. These data support the notion that heterogeneity observed between synovial tissues is reflected in the FLS as a stable trait. Clearly, the list of genes that are differentially expressed between the tissue and FLS subgroups facilitates our understanding of the pathophysiology of the molecular forms of disease. Moreover, the differences in the gene expression profiles reflect important aspects of biological variation within the clinically diagnosed disease that provide a molecular basis for the well recognized but as yet poorly understood heterogeneity in RA and may help to (sub)classify rheumatic diseases.

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6

Hyper-ERAD: a novel pathomechanism for rheumatoid arthritis

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Rheumatoid arthritis (RA) is a joint-affecting disease and is characterized with overgrowth of articular synovial cells, so-called 'pannus'. To understand the pathomechanism of RA, we attempted to characterize the rheumatoid synovial cell and found a novel protein, 'Synoviolin (synovial cell + protein)'. Structural analysis indicated that Synoviolin is an endoplasmic reticulum (ER)-resident E3 ubiquitin ligase, which is important for 'ER-associated degradation (ERAD)'. Its overexpression causes arthropathy, resembling RA in mice. Moreover, the heterozygote of Synoviolin (+/–) is remarkably resistant to collagen-induced arthritis. These 'gain of function' and 'loss of function' analyses clearly indicate the important role of Synoviolin in arthropathy.

Therefore, we present a new pathogenic mechanism for RA that is related with ERAD, an essential system for ER homeostasis by eliminating unfolded proteins from the organelle. Recent studies have shown that the accumulation of unfolded proteins mediated by impaired ERAD system results in ER stress-induced apoptosis of cells and causes various degenerative human diseases. Besides, the ERAD system is tightly linked with cell growth, apoptosis and differentiation. This evidence led us to a new hypothesis that 'hyper function of ERAD system' may cause synovial cell overgrowth through its anti-apoptotic effect in RA. Thus, we propose that inhibition of Synoviolin is one of the promising strategies for RA treatment by targeting synovial cells.

Session II – Role of leptin and eicosanoids in arthritic diseases

7

A role for leptin in immune-mediated inflammatory diseases?

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Leptin, the product of the Ob gene, is a polypeptide hormone that plays an important role in the regulation of body weight by inhibiting food intake and stimulating energy expenditure. Moreover, leptin exhibits a variety of other effects including the regulation of endocrine function, reproduction and hematopoiesis. Consistently, leptin-deficient mice are not only obese, but display major hormonal disturbances, including hypercorticosteronemia, diabetes, and infertility. In addition, it has been known for many years that leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice have an altered immune response. More recent studies have shown that T cells and B cells express the long signaling leptin receptor isoform (ObRb) and that leptin exerts direct effects on T lymphocytes, including the stimulation of cell proliferation, the promotion of Th1 responses, and the protection of thymocytes from corticosteroid-induced apoptosis. Consistently, ob/ob mice are protected from inflammation mediated by T cells and B cells in some models such as experimental colitis, experimental autoimmune encephalomyelitis, and concanavalin A-induced hepatitis. Conversely, the administration of recombinant leptin increased the severity of experimental autoimmune encephalomyelitis. Using a model of antigen-induced arthritis, we demonstrated that ob/ob and db/db mice have a milder form of arthritis and that cellular and humoral immune responses to the injected antigen are decreased as compared with wild-type mice. Furthermore, *ex vivo* stimulated lymph node cells from ob/ob and db/db mice produced less interferon gamma and more IL-10 than cells obtained from control mice.

Leptin production is regulated by various proinflammatory cytokines during the acute-phase response, and leptin stimulates the production of both proinflammatory and antiinflammatory cytokines *in vitro*. ob/ob mice exhibit increased susceptibility to lipopolysaccharide-induced and tumor necrosis factor alpha-induced lethality, suggesting that leptin is also involved in regulating the innate immune response. We recently examined the role of leptin in zymosan-induced arthritis (ZIA), where joint inflammation is dependent on innate immunity. Our results demonstrate that, as opposed to antigen-induced arthritis, ZIA is not impaired in ob/ob and db/db mice. On the contrary, these mice exhibit a delayed resolution of the inflammatory process, with increased circulating levels of IL-6 and serum amyloid A, and a tendency to develop more severe joint damage. Leptin deficiency thus appears to interfere with adequate control of the inflammatory response in ZIA.

Although data obtained in different models of inflammatory diseases suggest that leptin plays a role in immune response, the analysis of direct effects of leptin on lymphocytes *in vivo* is precluded by the

important hormonal and metabolic alterations, which are linked to leptin deficiency. To our knowledge, it has never been thoroughly investigated to which extent immune defects in ob/ob or db/db animals are linked to leptin deficiency or, respectively, leptin receptor deficiency per se, as opposed to confounding factors such as hypercorticosteronemia. To examine the importance of direct effects of leptin on lymphopoiesis and immune response, we generated bone marrow chimeras by transplantation of db/db bone marrow cells into lethally irradiated normal recipient mice, which provide a normal environment for the grafted cells. Donor db/db mice display a marked atrophy of the thymus as compared with db/+ controls. In bone marrow chimeras, the size and cellularity of the thymus were not different between mice grafted with db/db or db/+ bone marrow 12 weeks after the graft, suggesting that the thymic atrophy observed in db/db donors is not due to direct effects of leptin on lymphocytes. Moreover, RT-PCR analyses suggest that CD4/CD8 double positive thymocytes do not express ObRb. Further studies are in progress in order to examine which cell types respond to leptin in the thymus and whether impaired leptin signaling in lymphocytes affects immune responses after antigen challenge in db/db bone marrow chimeras.

8

Is leptin the link between obesity and osteoarthritis?

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Leptin is a small, 16-kDa protein produced and secreted primarily by adipocytes found in white adipose tissue. Initially discovered as a central regulator of appetite and energy expenditure, leptin may also be involved in the regulation of metabolic activity in the growth plate and bone. Since obese individuals have an increased incidence of osteoarthritis (OA), we evaluated the contribution of leptin to cartilage changes associated with this degenerative disease.

Leptin levels in synovial fluid samples obtained from OA patients undergoing joint replacement were measured by ELISA assay. In addition, histologic sections of tibial plateau cartilage and osteophytes obtained from OA patients were graded using the Mankin score, and were immunostained using antibodies to leptin, transforming growth factor beta 1 (TGF-β1), and insulin-like growth factor 1 (IGF-1). For experimental studies, various doses of leptin (10, 30,100, and 300 μg) were injected into the knee joints of rats. Tibial plateaus were collected and processed for proteoglycan synthesis by radiolabeled sulfate incorporation, and for expression of leptin and growth factors by RT-PCR and immunohistochemical analysis.

Our results indicated that leptin was observed in synovial fluid from the human OA-affected knee ($n = 14$ men, 8.16 ± 5.5 μg/l; and $n = 6$ women, 12.95 ± 8.92 μg/l). These leptin concentrations were correlated with the body mass index ($r = 0.572$, $P < 0.01$). Interestingly, a marked expression of leptin was observed in OA cartilage, especially in fibrillated cartilage with clusters and in osteophytes, while few chondrocytes produced leptin in normal cartilage. Furthermore, the pattern and level of leptin expression were related to the grade of cartilage destruction, and paralleled those of growth factors (IGF-1 and TGF-β1). When injected into rat knee joints, leptin stimulated chondrocytes anabolic functions and induced the synthesis of IGF-1 and TGF-β1 in cartilage at both mRNA and protein levels.

Taken together, these findings provide a new peripheral function to leptin as a key regulator of chondrocyte metabolism, and indicate that leptin may play an important role in the pathophysiology of OA.

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Regulation of eicosanoid synthesis by leptin

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Leptin is an adipocyte-derived hormone that is found in the peripheral circulation in correlation with total body fat mass. While leptin is best known for its ability to regulate energy homeostasis, it is a pleotropic hormone whose receptor is widely distributed in various tissues including cells of the immune system. We have previously reported that leukotriene (LT) synthesis in macrophages from leptin-deficient mice is attenuated and that the provision of exogenous leptin restores this defect. To explore the possibility that leptin regulates eicosanoid synthesis in cells from normal animals, we cultured rat alveolar and murine peritoneal macrophages overnight with media alone or with increasing doses of leptin. Following stimulation with calcium ionophore (A23187) or zymosan, we observed that leptin enhanced prostaglandin E_2 and LT synthesis in a dose-dependent manner. Since arachidonic acid (AA) is the metabolic precursor of the eicosanoids, we next asked whether leptin regulates AA release and phospholipase A_2 (PLA_2) activity. Rat alveolar macrophages (AMs) were prelabeled overnight with [^{3}H]AA and cultured with increasing doses of leptin on the following day. Leptin enhanced A23187-stimulated [^{3}H]AA release in a dose-dependent manner that peaked at 5 ng/ml (300% above control). Using rat AMs pretreated overnight with leptin, we observed that leptin enhanced both calcium-dependent and calcium-independent PLA_2 activity. To determine the time required for leptin to enhance AA release, AMs were prelabeled overnight with [^{3}H]AA, cultured with leptin (5 ng/ml) on the following day, and cell culture medium was collected following stimulation with A23187. Leptin enhanced AA release at 30 min, and 1, 2, 4, and 16 hours following A23187 stimulation. To explore the mechanism by which short-term leptin pretreatment enhances AA release, we assessed $cPLA_2\alpha$ phosphorylation in rat AM lysates following leptin pretreatment. Western blot analysis of immunoprecipitated $cPLA_2\alpha$ revealed that leptin pretreatment of rat AMs increased $cPLA_2\alpha$ phosphorylation (ser505) in a dose-dependent manner that peaked at 15 min (sevenfold increase with 10 ng/ml) before returning to baseline levels at 60 min. To determine the mechanism by which long-term leptin pretreatment enhances macrophage AA release and LT synthesis, we assessed the expression of 5-lipoxygenase, 5-lipoxygenase activating protein, and $cPLA_2\alpha$ in murine peritoneal macrophages and of $cPLA_2\gamma$ in rat AMs using western blot analysis. Overnight pretreatment with leptin increased the expression of 5-lipoxygenase (200%) in murine peritoneal macrophages and of $cPLA_2\gamma$ (180%) in rat AMs. Our data suggest that the increased incidence of inflammatory diseases, such as asthma and osteoarthritis, associated with obesity may be related to the ability of leptin to increase AA release and eicosanoid synthesis.

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Transcriptional regulation of the mPGES-1 gene in primary cultured articular chondrocytes

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Healthy cartilage is maintained in a state of dynamic equilibrium by matrix synthesis and matrix degradation by the chondrocytes. Any dysregulation with increased degradation and/or inadequate synthesis leads to hte loss of tissue structure and function, as in rheumatoid arthritis and osteoarthritis. The proinflammatory cytokine IL-1 plays a major role in this phenomenon. IL-1 acts on chondrocytes in part by stimulating the release of prostaglandin E_2 (PGE_2) at the sites of inflammation. Recently, a human membrane-associated prostaglandin E_2 synthase-1 (mPGES-1) was cloned. This enzyme catalyzes the con-

version of prostaglandin H_2 to PGE_2 in a highly specific manner. We previously demonstrated that mPGES-1 mRNA is induced by IL-1 in chondrocytes in a dose-dependent and time-dependent manner.
In order to study the transcriptional regulation of mPGES-1 in primary rabbit articular chondrocytes, we have cloned its promoter upstream of the CAT ORF (vector pCAT3-basic; Promega, Charbonnières-les-Bains, France). We show by transient transfection experiments that the mPGES-1 promoter is stimulated by IL-1. A close examination of putative binding sites has revealed the presence of two CCAAT/Enhancer Binding Protein (C/EBP) binding sequences (TTNNGNAAT) located between −548 to −558 base pairs and −610 to −619 base pairs. Co-transfections of expression vectors encoding the two different isoforms of C/EBP (β and δ) strongly stimulate the promoter activity. To further study the role of C/EBP in mPGES-1 expression, we performed gel shift experiments on wild-type and mutated oligonucleotides derived from the mPGES-1 sequence. These experiments confirm the specific binding of C/EBP on the mPGES-1 promoter. Taken together, our results suggest that C/EBP factors indeed bind and regulate the mPGES promoter in articular chondrocytes.

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ALXR, a common GPCR gateway for antiinflammatory and/or proinflammatory cell activation: relevance of selective kinase and phospholipase signaling by uteroglobin, lipoxin A_4 and serum amyloid A in ALXR-mediated chemotaxis

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The lipoxin A_4 receptor (ALXR) is expressed in fibroblast-like synoviocytes and is targeted by both antiinflammatory and proinflammatory endogenous ligands. Recently, annexin-1 (lipocortin) has been identified to be an ALXR ligand. Uteroglobin (Ug) is secretoglobin that shares with annexin-1 properties such as potent inhibition of phospholipase A_2 (PLA_2) as well as sequence similarities in the 'antiflammin' consensus region. Potential receptor interactions of Ug with ALXR were therefore investigated and PLA_2 activity assays were performed in CHO and HL-60 cell lines stably overexpressing ALXR. Serum amyloid A (SAA), an ALXR ligand displaying proinflammatory activities, was compared with two antiinflammatory ligands, Ug and lipoxin A_4 (LXA_4). While Ug and LXA_4 did not alter baseline PLA_2 activity (Ug) or provoked a pulsed peak of activity (LXA_4), SAA did induce a delayed and prolonged activation of PLA_2. SAA-induced PLA_2 activation was sharply inhibited in the presence of Ug. Ug inhibition of SAA-dependent phospholipase activity occurred at submillimolar Ca^{2+} concentrations, suggesting an effect on cytosolic PLA_2 (rather than secretory PLA_2, a known target inhibited by Ug and annexin-1 but requiring millimolar Ca^{2+}). Studies of Erk, p38 and AKT kinase phosphorylation confirmed that SAA and Ug interact with ALXR to oppositely regulate GPCR-coupled downstream signaling events. Overall the analysis of phospholipase D activity, kinase phosphorylation and cAMP levels also suggested that proinflammatory and antiinflammatory ligands of ALXR engage differently coupled pathways, leading to activation of these processes (SAA) or to their inhibition (LXA_4 and Ug). These signaling events are functionally matched by the ability of SAA to stimulate NF-κB activity, IL-8 release and cell chemotaxis, and that of LXA_4 and Ug to strongly inhibit them. Expression of Ug and SAA mRNAs was also detected in human fibroblast-like synoviocytes, suggesting that ALXR may play a pivotal role in the pathophysiology of arthritis. In addition, annexin-1 and Ug inhibition of PLA_2 offers enticing new venues to control inflammatory arthritides by limiting, for example, cascade signaling amplification via synthesis of proinflammatory eicosanoids, along with a redirection of ALXR signaling toward antiinflammatory feedback mechanisms.

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Regulation of neutrophil trafficking by the lipid mediators of inflammation

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Background The lipid mediators of inflammation include platelet-activating factor (PAF) and several classes of metabolites of arachidonic acid derived from the cyclooxygenase (prostaglandins and thromboxanes) and lipoxygenase pathways, such as leukotrienes (LTs) and lipoxins. Of these lipid mediators, LTs and PAF are potent agonists of leukocytes and endothelial cells and show important proinflammatory properties. Several elegant studies have recently provided strong support for a role of one of these mediators, LTB_4, in animal models of rheumatoid arthritis [1–3]. Interestingly, LTB_4, LTC_4/LTD_4 and PAF are concomitantly generated at the blood–endothelium interface when neutrophils bind to activated endothelial cells at inflammatory sites *in vivo* and are exposed to a chemoattractant. The hypothesis explored in this project is that the three classes of lipid mediators collectively and synergistically act to promote and facilitate neutrophil extravasation and accumulation at inflammatory sites. The hypothesis also implicates that, given the high level of redundancy in the abilities of the three lipid mediators to activate neutrophils and endothelial cells, the blockade of one of these mediators can only have a modest and incomplete inhibitory effect on neutrophil extravasation.
Objective The objective of these studies is therefore to compare the role of each individual mediator and the collective effect of the three mediators in regulating neutrophil trafficking. Another objective is to assess whether or not the combined use of antagonists to the three classes of mediators could result in a stronger inhibitory effect on neutrophil trafficking.
Methods The studies are performed using potent and selective LTB_4, LTD_4 and PAF antagonists in both *in vitro* and *in vivo* models. The separate and collective role of the three classes of lipid mediators will be investigated in the migration of human neutrophils through a monolayer of human endothelial cells grown on a gel of extracellular matrix proteins. The studies also involve the use of models of dermal inflammation in rats and rabbits and the mouse air pouch model.
Results The results show that, in the various models tested, neutrophil migration elicited by proinflammatory agents such as IL-1, tumor necrosis factor alpha and lipopolysaccharide is generally decreased by LTB_4 or PAF antagonists, and that the combination of both antagonists results in a greater inhibitory effect, supporting the concept that several lipid mediators generated locally (at the blood–endothelium interface and/or at inflammatory sites) regulate neutrophil trafficking. Additional experiments involving antagonists to the three classes of lipid mediators, as well as LTB_4 and PAF receptor knockout mice, are in progress.

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Session III – Autoimmunity and inflammation: from toll to reg

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The nonsense allele *oblivious* reveals a sensor of di-acylglycerides acting in conjunction with TLR2 and TLR6

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The mammalian Toll-like receptors (TLRs) activate cells of the innate immune system when stimulated by diverse ligands of microbial origin. In some instances, these ligands are directly engaged by the TLRs; however, this is not necessarily true in all cases. TLR2 recognizes multiple, structurally disparate microbial ligands, consistent with a requirement for co-receptors in ligand binding. Using *N*-ethyl-*N*-nitrosourea, we generated the recessive immunodeficiency phenotype *oblivious*, in which macrophages show diminished awareness of the S-enantiomer of the di-acylated bacterial lipopeptide MALP-2 and lipoteichoic acid, together with spontaneous ocular colonization by Gram-positive organisms and hypersusceptibility to *Staphylococcus aureus* infection. *Oblivious* macrophages readily detect the tri-acylated bacterial lipopeptide PAM_3CSK_4 as well as zymosan, revealing that some TLR2 ligands are activated via an *Oblivious*-independent pathway. The gene responsible for the *oblivious* phenotype has been positionally cloned. In its ability to carry the lipoteichoic acid and MALP-2 signal to the transmembrane signaling receptors TLR2 and TLR6, *Oblivious* serves a function analogous to CD14, which concentrates the lipopolysaccharide signal for transduction by TLR4. Besides microbial molecules, *oblivious* also serves as a receptor for endogenous molecules and may mediate (some) of the inflammatory events involved in the development of atherosclerosis.

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An apoptotic signaling pathway activated by Nod1

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Nod1 and Nod2 are two cytosolic proteins thought to play a role in innate immunity. Both detect the presence of microbes through recognition of peptidoglycan fragments but also may initiate apoptosis. The *nod2* gene has been strongly associated with several autoimmune diseases and particularly Crohn's disease; in contrast, *nod1* polymorphisms have not been linked to any genetic disorders. Here we provide multiple lines of evidence showing that Nod1 participates in apoptosis. Nod1-deficient breast cancer cells (MCF-7) were more resistant to tumor necrosis factor-induced cytotoxicity, and this was accompanied by a reduction in caspase signaling. Further, γTriDAP, a naturally occurring tripeptide product from peptidoglycan and a ligand for Nod1, induced cell death consistent with apoptosis in wild-type MCF-7 cells but not in Nod1-deficient cells. γTriDAP triggered processing of PARP and many caspases, including caspase 6, caspase 7, caspase 8 and caspase 9. Only caspase 9 inhibitor totally abrogated γTriDAP cytotoxicity. RIP2/RICK, a downstream protein kinase of Nod1, appears to be an essential component of the Nod1 proapoptotic pathway since expression of a dominant negative form of RIP2 abolished γTriDAP-induced cell death. This is a newly defined activity for Nod1 and suggests that Nod1-induced apoptosis may be responsible for cell injury in a variety of disease states.

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Post-transcriptional regulation of tumor necrosis factor alpha expression

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The success of tumor necrosis factor (TNF) antagonists in the therapy of inflammatory arthritides has established the central role of this cytokine in the pathogenesis of these disorders. TNF is notable in that it is predominantly controlled at the post-transcriptional level in macrophages. TNF biosynthesis is highly regulated by the AU-rich element (AURE) found in its 3′ untranslated region. This AURE regulates both the stability and rate of translation of TNF mRNA. In the macrophage, the ERK, JNK, and p38 SAPK signaling pathways converge on the AURE in regulating the nuclear export, stability and translation of TNF mRNA. Second, the C3H zinc finger protein, tristetraprolin (TTP), appears to play a major role in the post-transcriptional regulation of TNF by binding the AURE. Recent studies have identified that TTP is a target of the p38 SAPK/MAPKAP K2 kinase (MK2) pathway. Phosphorylation of TTP by MK2 has been reported *in vitro* and *in vivo*; data suggest that MK2 activation inactivates the function of TTP as a destabilizing protein.

Intriguingly, TTP binding to the AURE does not appear to be regulated by this phosphorylation. Rather, the function of TTP seems to be modulated through interactions with specific proteins that alter its subcellular localization. This creates a model where TTP provides specificity in binding cytokine-type AURE but the consequences of this interaction are determined by protein–protein interactions. Second, we have identified that TTP does not bind all AURE, but rather exhibits specificity for nUAUUUAUn sequences. Third, we have identified that TTP regulates its own mRNA stability. Fourth, we have demonstrated that TTP localizes to the polysomes in the context of macrophage activation by lipopolysaccharide. Fifth, we have demonstrated that TTP is expressed in many different hematopoietic cells and appears to function as an AURE binding protein. Thus, although the role of TTP in TNF biology is best understood in the macrophage, it appears that some, if not all, of these concepts may be relevant to other cells involved in the immune and inflammatory response.

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A proinflammatory chemokine, CCL3, sensitizes the heat-gated and capsaicin-gated ion channel, TRPV1

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Pain, a critical component of host defense, is one of the hallmarks of the inflammatory response. We therefore hypothesized that pain might be exacerbated by proinflammatory chemokines. To test this hypothesis, CCR1 was co-transfected into HEK293 cells together with the Vanilloid Receptor 1 (TRPV1), a cation channel required for certain types of thermal hyperalgesia. Capsaicin induced calcium influx by TRPV1. When CCR1:TRPV1/HEK293 cells were pretreated with CCL3, the sensitivity of TRPV1-mediated calcium flux was increased about fivefold. Pertusis toxin inhibited CCL3-elicited sensitization of TRPV1, indicating the involvement of G-protein signaling. RT-PCR analysis data showed that a spectrum of chemokine and cytokine receptors are expressed in rat dorsal root ganglia (DRG). Immunohistochemical staining of the DRG showed that CCR1 coexpressed with

TRPV1 on over 85% of small diameter neurons. CCR1 on neuronal cells was functional, as demonstrated by CCL3-induced calcium flux and protein kinase C activation. Pretreatment with CCL3 enhanced the response of DRG neurons to capsaicin, and this sensitization was inhibited by pertussis toxin, U73122, or staurosporine. Futhermore, injection of CCL3 into mice spine cords enhances the sensitivity of the mice tails toward the hot water, indicative of chemokine-induced sensitization effects *in vivo*. The fact that a proinflammatory chemokine, by interacting with its receptor on small-diameter neurons, sensitizes TRPV1 reveals a novel mechanism of receptor cross-sensitization that may contribute to hyperalgesia during inflammation.

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A genetic analysis of lupus

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Systemic lupus erythematosus is a complex multigenic inherited disease with susceptibility determined by a combination of genetic, environmental and stochastic factors. Although not yet defined, recent technical advances have provided the means to dissect the component genetic contributions of polygenic traits. We have applied such approaches to mouse models of spontaneous systemic lupus erythematosus, and in this presentation I will summarize our genome-wide mapping studies that identified loci predisposing to several major lupus-related traits. Through the generation and study of interval congenic lines, precise mapping, and screening of candidate genes, identification of the specific genes and mechanisms associated with some of the major loci is currently being pursued.

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Cellular-based immune modulation in arthritis

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While the symptoms associated with rheumatoid arthritis (RA) are well described, the factors that are crucially involved in induction and/or progression of RA are poorly defined. Nonetheless, it is generally accepted that cells belonging to the adaptive immune system (i.e. B cells and T cells) are intimately engaged in the processes responsible for RA induction and/or progression. As these cells require triggering of their antigen receptors before they can exert their functions, it is likely that antigen recognition is important in the pathological processes driving RA. Unfortunately, no antigens casually related to the induction/perpetuation of RA are known. Nonetheless, studies addressing the question of how immunity against autoantigens is regulated, combined with studies aiming at defining the molecular identity of antigens involved in disease, will be important to gain a better understanding of the pathways involved in the induction/progression of RA.

Recently, we have found that $CD4^+CD25^+$ regulatory T cells play a pivotal role in the control of arthritis in mice. More importantly, treatment of established arthritis by adoptive transfer of these cells stopped further progression of disease. These data indicate the feasibility to inhibit ongoing disease by specific manipulation of the immune system. The data described also indicate that the class of immune response against autoantigens could profoundly influence the susceptibility for, and/or outcome of, autoimmune responses. However, until now, there has been only limited evidence for autoantigen-specific regulatory responses in humans. Here we analyzed the natural immune response against Human Cartilage gp39 (HC-Gp39), a candidate autoantigen in RA. Peripheral blood mononuclear cells from healthy individuals reacted against HC Gp39 with the production of IL-10, but not interferon gamma. *Ex vivo* assays indicated that the natural occurring HC Gp39-directed immune response in bulk is capable of suppressing cytotoxic T-cell and recall responses, indicating that, rather than being unresponsive, the HC Gp39-directed immune response in healthy individuals is biased towards a regulatory phenotype. Moreover, $CD4^+$ T-cell lines directed against HC Gp39 expressed CD25, GITR, CTLA-4 and Foxp3 molecules and were capable of suppressing other immune responses. Cell–cell contact was required for this suppression. In contrast, the quality of the HC Gp39-directed immune response in patients with RA exhibits polarization toward a proinflammatory Th1 phenotype. Together these findings indicate that the presence of HC Gp39-specific immune responses in healthy individuals may have a profound inhibitory effect on inflammatory responses in areas were HC Gp39 is present, and imply that the balance of autoreactive proinflammatory and antiinflammatory HC Gp39-directed immune responsiveness is disrupted in RA patients.

Plenary lecture – The use of tissue engineering for joint structure repair: a future?

Current tissue engineering approaches to cartilage repair

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Chondral defects, generated as a result of trauma and injury or degenerative joint diseases, such as osteoarthritis, represent some of the most challenging orthopedic conditions in terms of natural tissue healing and repair. Damages to the articular cartilage fail to elicit significant reparative activity, due to the acellularity of the tissue. Clonal proliferation of articular chondrocytes is often seen, and results in the production of mechanically inferior fibrocartilage. Extensive degeneration of the articular surface eventually necessitates total joint arthroplasty. There is, therefore, a timely need for the development of biological approaches to cartilage repair. The emerging research discipline of tissue engineering aims to develop functional tissue substitutes by combining experimental approaches in biology and engineering, and represents a particularly attractive technology for the treatment of skeletal diseases, most of which involve tissue degeneration or failure to heal. Functionally, it is instructional to approach cartilage regeneration in the context of developmental chondrogenesis; that is, the formation of cartilage from a progenitor mesenchymal cell population during embryonic development. In particular, adult tissue-derived mesenchymal stem cells (MSCs), which display multilineage differentiation potential, are currently considered a highly promising source of progenitor cells for tissue engineering. Chondrogenesis in the developing vertebrate limb consists of a highly coordinated and orchestrated series of events involving the commitment and differentiation of mesenchymal cells to mature chondrocytes. This process is regulated by the sequential and coordinated expression of genes that encode for specific cell adhesion molecules, growth factors, and extracellular matrix molecules that carry out morphogenetic and signaling activities. Recently, members of the Wnt family of signaling molecules have been functionally implicated in limb development. The Wnts comprise a large family of cysteine-rich glycoproteins that perform a number of inductive and regulatory functions in both normal development and oncogenic transformations. The expression of various Wnts has been identified in the developing limb, and our recent studies showed that Wnts play an important role in mesenchymal chondrogenesis. Interestingly, our recent work with human MSCs indicates the functional involvement of Wnts and the cell adhesion molecular, N-cadherin, as well as members of the transforming growth factor beta superfamily in MSC chondrogenesis, suggesting that regenerative chondrogenesis and developmental chondrogenesis are likely to share common regulatory pathways. By combining MSCs, biodegradable polymeric scaffolds, and the application of growth and differentiation factors, we are currently developing cartilage constructs *in vitro* that are potentially applicable for cartilage repair *in vivo*. Specifically, we recently fabricated a nanofibrous scaf-

fold using a synthetic biodegradable polymer, poly(ε-caprolactone), and demonstrated its ability to support *in vitro* chondrogenesis of MSCs. The electrospun porous scaffold consisted of uniform, randomly oriented nanofibers (700 nm diameter); MSCs seeded into the scaffold and cultured in the presence of transforming growth factor beta 1 differentiated to chondrocytes, indicated by gene expression and histological profiles, at a level similar to that observed in high-density pellet cultures. The physical nature and improved mechanical properties of such scaffolds, particularly in comparison with cell pellets, suggest that these constructs may serve as a practical carrier for MSC transplantation in cell-based tissue engineering approaches to cartilage repair, supported by our preliminary animal model study. The future success of cartilage tissue engineering is dependent on a number of requirements, including expansion of progenitor cells, optimization of biomaterial scaffold design, and molecular enhancement of cell differentiation and growth using biologics and gene therapeutic means. We believe that the understanding of developmental chondrogenesis serves as a rational and powerful paradigm for cartilage tissue engineering.

Session IV – Novel therapeutic strategies: don't miss the target

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Regulating innate immune response cytokines in synovitis

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Several studies have provided powerful proof of concept for the notion that single cytokine targeting can manifest effective immune suppression even in the context of complex networks of cytokine activities. We have studied the biology and functional expression of the innate response cytokine IL-15 and, more recently, members of the IL-1/IL-1 receptor superfamily in inflammatory synovitis. These cytokines are characterised by early upregulation during inflammatory responses and characteristic functional synergy and cross-regulation in the intact immune response. IL-15 is an IL-2-like cytokine of 15 kDa that possesses broad proinflammatory activities via its heterotrimeric receptor (IL-15Rα/IL-15/2Rβ/common γ), including promoting T-cell maturation, activation, and cytokine production, natural killer cell maturation and effector function, neutrophil activation, dendritic cell activation, endothelial cell rescue from apoptosis, macrophage activation and B-cell activation and isotype switching. IL-15 is expressed at mRNA and protein levels in several inflammatory arthritides including rheumatoid arthritis. When targeted in rodent models of inflammation using either soluble IL-15Rα or modified IL-15 mutant fusion proteins, amelioration of onset and of existing arthritis is observed. A recent phase I study in which a fully human IgG_1 monoclonal antibody (HuMax-IL15; Genmab, Copenhagen, Denmark) was administered to 30 patients with active rheumatoid arthritis indicated that IL-15 blockade was well tolerated up to 8 weeks, with early indications of efficacy detected. We have now observed close interactions between IL-15 and IL-1 receptor superfamily signalling in promoting T-cell activation. Specifically, IL-15 synergises with the TLR2 ligand BLP to promote memory T-cell activation (co-stimulatory) and also via a cell membrane-dependent pathway to promote adjacent macrophage activation and tumour necrosis factor release. We have also observed that a further member of the IL-1 receptor superfamily, namely ST2, is expressed in synovial fibroblasts. Membrane-bound ST2 negatively regulates type I IL-1 receptor and TLR4 signalling by sequestrating the adaptors MyD88 and Mal. Intriguingly, soluble ST2 suppresses development of collagen-induced arthritis in DBA/1 mice if given prophylactically, and also established collagen-induced arthritis if administered after disease onset. Together these data suggest that complex interactions between the common γ-receptor chain and IL-1 receptor superfamily signalling cytokines are of importance in developing synovial inflammatory responses and that elucidation of these pathways offers therapeutic utility.

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Galectin-3 in osteoarthritis: from a protective to a destructive role

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Background Osteoarthritic (OA) chondrocytes are able to re-express numerous genes normally activated in the growth plate, and more particularly in the hypertrophic zone. Among several genes, we are interested in studying galectin-3 (gal-3) since we have recently demonstrated that its expression was increased in OA cartilage [1]. gal-3 is a mammalian lectin, which interacts with β-galactoside residues and is involved in numerous functions such as adhesion, splicing activity, cell cycle regulation, as well as a receptor for advanced glycation end products (AGE receptor). These functions are related to the gal-3 cellular localization. Indeed, this protein may be found in the plasma membrane, in cytoplasm and in the nucleus.

Objective In the present study, we investigated the role(s) of gal-3 using both the mono-iodoacetate-induced OA model and *in vitro* experiments.

Methods OA was induced by a single injection of iodoacetate (5 mg/ml, 2 µl) into each knee joint of 4-month-old mice (WT) or gal-3 null mice (KO). Mice were sacrified 7, 14 and 21 days after the single injection. Histologic evaluation was performed on sagittal sections of mouse knee joint. The severity of the OA lesions was graded on a scale of 0–14 in a blinded fashion, by two independent observers, using the histologic/histochemical scale of Mankin. Intracellular and extracellular roles of gal-3 were investigated in both human chondrocytes and in chondrogenic ATDC5 cells, a mouse cell line derived from the 129 strain.

Results Intra-articular injection of mono-iodoacetate, which induced osteoarthritis, upregulated the expression of gal-3 in WT mice 7 days post injection, reaching a statistical significance 14 days post injection ($P < 0.05$). The histologic grading score indicated that KO mice (control group) had a poorer quality of cartilage compared with WT mice (control group). Moreover, the induction of OA in KO mice showed a marked decreased of bone area, noticeable 7 days post injection ($P < 0.05$).

According to the results obtained, it seemed that gal-3 was important for the cartilage homeostasis. Colnot and colleagues have suggested that gal-3 could be implicated in chondrocyte survival [2]. Therefore, we treated OA chondrocytes with sodium nitroprusside (SNP), which is known to generate chondrocyte cell death. Our results showed that gal-3 was much further decreased than was Bcl2 in experiments performed under the same conditions [3]. Moreover, SNP decreased the gal-3 phosphorylation, which is a key process in the capacity of gal-3 to prevent cell death. Finally, ATDC5 cells transfected with a gal-3-expressing vector were more resistant to SNP-induced cell death compared with those transfected with the empty vector. On the other hand, Ohshima and colleagues found gal-3 in synovial fluid, particularly during inflammation [4]. Therefore, we investigated the potential role of exogenous gal-3 in chondrocyte cultures. Surprisingly, we found that exogenous gal-3 induced chondrocyte death.

One of the most fascinating phenomena is the regulation of gal-3 secretion. Indeed, several cells produced gal-3 but not all are able to secrete a great amount of it, chondrocytes belonging to the latter category. Conversely, gal-3 is secreted in a much greater quantity by inflammatory cells that could affect – at least locally (i.e. at the pannus level) – chondrocyte survival.

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Angiogenesis and arthritis: a potential therapeutic target?

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Musculoskeletal disorders such as rheumatoid arthritis (RA) are a common cause of pain and disability. Over the past decade, advances in understanding RA pathogenesis, based on studies of human cells and animal models of disease, have led to the identification of new targets for therapeutic intervention. Nevertheless, despite the clinical success of anticytokine biologicals, further initiatives in understanding RA pathogenesis to aid further drug discovery are highly desirable.

An early event in RA is an alteration in synovial blood vessel density. The hyperplasia of the synovium necessitates a compensatory increase in the number of blood vessels to nourish and oxygenate the tissue. A key response to increased requirement for nutrients and/or reduced oxygen is to form new blood vessels (angiogenesis). In RA, the synovial blood vessel number has been found to correlate with hyperplasia, mononuclear cell infiltration and indices of joint tenderness. Endothelial cells lining blood vessels within RA synovium have been shown to express cell-cycle antigens. A variety of angiogenic mediators, including cytokines and growth factors, have been identified in rheumatoid joints. We and other workers have shown that expression of the angiogenic factor vascular endothelial growth factor (VEGF) is increased in RA. Importantly, circulating levels of VEGF are elevated in patients with established RA and are reduced by anti-tumour necrosis factor alpha antibody treatment, suggesting that part of the beneficial effect of anti-tumour necrosis factor alpha in RA may be reduced angiogenesis.

However, new blood vessel formation may not keep pace with synovial proliferation leading to regions of hypoperfusion. Intra-articular oxygen tension is significantly lower in RA patients when compared with controls, and hypoxia-inducible transcription factors such as HIF-1α and HIF-2α are significantly upregulated. Of relevance in the context of RA, VEGF is significantly upregulated by hypoxia, which would promote further blood vessel formation. As well as being a key angiogenic factor, VEGF also promotes vascular endothelial cell survival, by increasing expression of anti-apoptotic proteins such as Bcl-2, and members of the inhibitor of apoptosis family (namely cIAP-1, XIAP and survivin), which directly bind to and inhibit caspases. Our data suggest that induction of these apoptosis inhibitors is mediated via the NF-κB pathway.

We are currently further investigating pathways activated by VEGF, as well as dissecting out the response of synovial cells to hypoxia, both in terms of induction of angiogenic molecules such as VEGF and upregulation of inflammatory cytokines such as tumour necrosis factor alpha. There have been a number of studies showing that angiogenesis blockade is effective in animal models of arthritis. Since VEGF is elevated early during the course of RA, and since RA is most active in the initial stages of disease, early treatment might reduce subsequent joint erosion and damage. RA may be a potential target for anti-angiogenic therapy, and targeting VEGF may prove to be especially beneficial.

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Mapping out the mitogen activated protein kinase pathway

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The mitogen activated protein (MAP) kinases represent another attractive target for rheumatoid arthritis (RA) because they can regulate cell proliferation, apoptosis, cytokine expression, and metalloproteinase production. They represent a complex, inter-related signal transduction mechanism that integrates extracellular stresses and induces an appropriate cellular response. The three major MAP kinase families, c-Jun-N-terminal kinase (JNK), extracellular regulating kinase (ERK) and p38 kinase, differ in their substrate specificity and subsequent responses to stress depending on the cell type and the environmental influences. The MAP kinases regulate various genes via both transcriptional and post-transcriptional mechanisms. The upstream MAP kinase kinases (MAPKK) serve as regulators of MAP kinase activity by phosphorylating specific threonine and tyrosine residues. MAPKKs are, in turn, regulated, by MAPKK kinases (MAPKKK or MAP3K).

The p38 MAP kinase is of particular interest and several inhibitors have progressed into clinical trials. There are at least four isoforms of p38 (α, β, γ and δ), although the α form is probably the most important in macrophages for cytokine regulation. Many of the cytokine regulatory effects appear to be mediated through the downstream p38 substrate MAPKAP-2. Some evidence suggests that tumor necrosis factor production, in particular, is regulated by this kinase. Preclinical models demonstrate that p38 inhibitors are effective in a number of animal models of arthritis, including murine collagen-induced arthritis. Of note, p38 inhibitors also suppress joint destruction in these models, perhaps due to a combination of indirect effects on cytokine expression and a direct effect on metalloproteinase production.

In vitro studies previously identified the MAPKKs MKK3 and MKK6 as the primary regulators of p38 phosphorylation and activation. To investigate a potential role for MKK3 and MKK6 in RA, we evaluated their expression and regulation in RA synovium and cultured fibroblast-like synoviocytes (FLS). Immunohistochemistry demonstrated that MKK3 and MKK6 are expressed in RA and osteoarthritis (OA) synovium. Digital image analysis showed no significant differences between OA and RA with regard to expression or distribution. However, phosphorylated MKK3/MKK6 expression was significantly higher in RA synovium and was localized to the sublining mononuclear cells and the intimal lining. Western blot analysis of synovial tissue lysates confirm the increased expression of phosphorylated MKK3/MKK6 in RA. Western blot analysis demonstrated constitutive expression of MKK3 and MKK6 in RA and OA FLS. Phospho-MKK3 levels were low in medium-treated FLS, but were rapidly increased by IL-1 and tumor necrosis factor alpha, although phospho-MKK6 levels only modestly increased. p38 co-immunoprecipitated with MKK3 and MKK6 from cytokine-stimulated FLS, and the complex phosphorylated ATF2 in an *in vitro* kinase assay. Studies using dominant negative constructs in cultured synoviocytes suggest that both are required for full activation of p38. These data are the first documentation of MKK3 and MKK6 activation in human inflammatory disease. By forming a complex with p38 in synovial tissue and FLS, these kinases can potentially be targeted to regulate the production of proinflammatory cytokine production in inflamed synovium.

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The αvβ3 integrin as a therapeutic target for inhibition of bone resorption

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An early feature of inflammatory arthritis is an increase in the number, size and activity of osteoclasts leading to the destruction of periarticular and subchondral bone and mineralized cartilage [1]. Osteoclastic resorption is regulated by a number of processes, including the proliferation, differentiation and recruitment of osteoclast precursors, the migration and activation of mature osteoclasts, and programmed cell death. Regulatory mechanisms involve soluble mediators, cell–cell and cell–matrix interactions. The latter involve integrins – heterodimeric receptors that link the cytoskeleton to the extracellular matrix and mediate outside-in and inside-out signaling. Mature osteoclasts express three integrins (αvβ3, αvβ1 and α2β1), with the αvβ3 vitronectin receptor predominating.

We used a single cell assay to demonstrate that macrophage-colony stimulating factor (M-CSF) and transforming growth factor beta are chemotaxins for mammalian osteoclasts. Interestingly, the RGD-containing disintegrin echistatin, which binds αvβ3 integrin, inhibited M-CSF-induced chemotaxis at concentrations as low as 0.1 nM [2]; whereas it did not inhibit migration induced by transforming growth factor beta [3].

We have recently examined the effects of Vitaxin®, a humanized monoclonal antibody that blocks human and rabbit αvβ3 integrins [4]. Vitaxin® caused a concentration-dependent decrease in the number of rabbit osteoclasts attached to plastic. Moreover, Vitaxin® inhibited resorption by rabbit osteoclasts cultured on slices of bovine bone (Vitaxin® at 0.7 nM reduced resorption by ~50%). Vitaxin® also suppressed bone resorption by *in vitro*-derived human osteoclasts, with a maximum of 63% inhibition observed at concentrations greater than 2 nM. Vitaxin® does not react with rodent αvβ3 integrin; therefore, we used a blocking anti-rat αvβ3 integrin to assess effects on rat osteoclast motility. This antibody induced rapid retraction of M-CSF-treated rat osteoclasts but, unlike echistatin, did not inhibit chemotaxis induced by M-CSF.

Evidence from our laboratories and others, using disintegrins, antibodies, RGD-containing peptides and small molecules that bind the αvβ3 integrin, point to its potential as a target for the development of antiresorptive therapies. The role of αvβ3 integrins in angiogenesis may make such therapeutics especially useful for the treatment of rheumatoid arthritis and metastatic bone disease. Differences in responses to various agents and to genetic ablation of αvβ3 integrins point to multiple underlying mechanisms and underscore the need for further investigations.

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Session V – Development of DMOADS: a long and risky business

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Issues in structure-modifying osteoarthritis drug development: new insights regarding radiographic clinical trial methods

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Background Over the past several decades, the osteoarthritis (OA) field has relied on radiographs in clinical trials, in order to assess longitudinal structural changes in weight-bearing joints such as the knee. Because alternative imaging modalities such as magnetic resonance imaging are beginning to mature, it is timely to compare and contrast the utility of these imaging methods for the purpose of conducting future structure-modifying OA drug clinical trials

Objective Insights from a large, multicenter knee OA study will be shared in the context of considering the next generation of longitudinal imaging methods to study OA.

Methods Patients in the study all had radiographic and symptomatic knee OA. A total of 2400 patients were randomized to the study, with an 85% study completion rate. The radiographic data for the study were collected at baseline, month 12 and month 24, using a highly standardized radiographic method directed at the medial compartment of the signal knee. Fluoroscopic confirmation of proper knee position was achieved at each study visit.

Results A 2-year, 2400-patient study of knee OA has been completed and is being analyzed. Insights will be shared at the GARN Conference.

Session VI – The use of imaging and biomarkers in the assessment and follow-up of arthritis

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Overview: imaging, biomarkers and arthritis

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The recent introduction of effective structure-modifying therapies for rheumatoid arthritis (RA) has changed the way that rheumatologists manage patients with RA, and this has created new demands on imaging and biochemical markers both in clinical practice and in clinical research. Among other things, it has shifted therapeutic strategy towards early, aggressive treatment before the onset of erosive joint damage in order to prevent irreversible functional disability. Additionally, it has made it unethical to withhold active therapy and therefore to perform true placebo-controlled clinical trials. This has necessitated using active comparator study designs instead, which require more patients, more clinical sites and longer studies to test the efficacy of putative new therapies. This adds time and cost to drug development, which slows progress and potentially raises the cost of new therapies that do get approved. Enriching study populations with rapidly progressing patients may offset some of this effect, but this necessitates the availability of prognostic markers that can accurately identify which patients are most likely to develop erosive damage and functional disability. This can be challenging in early RA, as 30% or more of the patients in early RA cohorts do not progress. Early prognosticators are also needed by clinical practitioners to determine which patients need aggressive treatment before the narrow window of opportunity for containing erosive disease closes.

Magnetic resonance imaging (MRI) provides greater sensitivity for bone erosions than radiography does, especially in early RA, when radiography is relatively uninformative. This, along with its ability to detect pre-erosive features, such as synovitis and bone edema, allows MRI to identify patients with the aggressive phenotype of RA very early in the course of their disease. Supplementing MRI with specialized molecular markers, such as CTX-I, CTX-II, glucosyl-galactosyl pyridinoline, COMP and others, may provide additional predictive power. These MRI and

molecular markers can also be used to monitor disease progression and treatment response in clinical trials with greater statistical power than radiography can offer.
Accordingly, as the introduction of structure-modifying therapy into mainstream clinical practice drives the demand for sensitivity and precision in predicting and monitoring erosive damage in early RA beyond the technical limits of conventional radiography, MRI and specialized molecular markers become increasingly attractive alternatives for evaluating therapeutic efficacy in clinical trials as well as in clinical practice.

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Imaging and osteoarthritis

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Understanding of joint cartilage development and the pathophysiology of osteoarthritis (OA) has previously been limited by the lack of a noninvasive method for assessing joint cartilage *in vivo*. There has been increasing interest in the use of magnetic resonance imaging (MRI) in the measurement of articular cartilage volume as a possible outcome measure in arthritis. Recent work has shown that the cartilage volume, as measured by MRI, can be measured in a valid and reproducible way, with a coefficient of variation of ~2%. The knee cartilage volume correlates inversely with the radiographic grade of OA. Studies have shown that significant loss in knee cartilage has already occurred by the time radiographic change occurs at the knee. Subjects with OA lose approximately 5% of their knee cartilage per annum, while normal, healthy males and females lose between 1% and 3% per annum. There is evidence from longitudinal data that loss of knee cartilage volume is associated with a worsening of knee symptoms and that those in the top tertile of rate of cartilage loss have a sevenfold increased risk of progressing to a knee replacement within 4 years. Other joint structures can be measured using MRI. Interesting data have emerged from examining knee bone marrow oedema and cartilage defects: bone marrow oedema has been shown to be associated with pain and progression of knee OA in the adjacent tibiofemoral compartment. Recent data suggest that knee cartilage defects predict healthy subjects at risk of losing knee cartilage and increase the risk of progressing to a knee replacement in those with knee OA. The emerging data suggest that MRI assessment of joints in normal subjects and those with OA has the potential to significantly increase our understanding of the pathogenetic mechanisms involved in the development of OA, and thus to identify strategies to prevent and treat this disease.

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A 2-year evaluation of disease progression by quantitative magnetic resonance imaging in a large primary osteoarthritic patient population

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The aim of this study was to expand our previous work assessing cartilage volume changes on knee osteoarthritic (OA) patients over time by quantitative magnetic resonance imaging (MRI).

A subset of 110 patients, chosen from a large clinical trial evaluating the impact of a bisphosphonate on OA knee, was studied. The overall patient population ($n = 1232$) represented a standard study with the mean age of 60.5 years, 61% female, and an average body mass index of 30.3 kg/m^2. Patients with KL Grade IV radiographs were excluded. MRIs of the knee were performed at baseline, 12 months and 24 months. The images were arranged randomly and quantified by readers blinded to the time sequence. Total cartilage volumes and cartilage from the medial and lateral compartments were analyzed. Data are presented for the population that had all three sets of MRIs ($n = 107$ patients). Data on cartilage volume changes with time are presented as pooled data.
The mean losses of knee OA cartilage volume in percentage from baseline, computed at all follow-up time points, were all statistically significantly different from zero and are similar to those previously obtained from a pilot study of 32 patients [1]. Three populations described as fast progressors ($n = 11$ patients; −13.2% of total cartilage), intermediate progressors ($n = 48$; −7.2%) and slow progressors ($n = 48$; −2.3%) were identified based on loss distribution at 24 months.

Table 1

Time point	Total cartilage*	Medial compartment*	Lateral compartment*
12 months	−3.7 ± 0.3%	−5.4 ± 0.4%	−2.2 ± 0.3%
24 months	−5.6 ± 0.4%	−8.1 ± 0.6%	−3.4 ± 0.4%

Data presented as mean ± standard error of the mean, $n = 107$. * $P < 0.0001$ for all measurements using a paired t test.

These data support our previous observations in a smaller cohort of patients, showing MRI to be a precise and sensitive measure, able to reliably detect significant changes in human knee OA cartilage volume over time; as early as 12 months. Moreover, the percentage loss of cartilage volume further validates, by its statistical power, the results of our pilot study.

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The use of imaging and biomarkers in the assessment and follow-up of arthritis

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The use of imaging allows an accurate definition of the pathogenesis of patients with inflammatory arthritis. It can identify certain predictive prognostic factors, such as erosions or the level of synovitis. By using three-dimensional imaging up to 10-fold greater sensitivity for the assessment of erosions is achieved. Using such methods it is possible to produce a more homogeneous population for entry into studies. The results of this are an improvement in outcome data with a consistency of response.
By directly imaging the site of pathology it is possible to increase both the sensitivity and validity of a study; for example, the level of synovitis can be used as a direct measure of outcome. Measuring erosions using ultrasound or magnetic resonance imaging can increase the sensitivity of studies that have as their endpoint structural damage. By increasing the power of studies it is possible to use smaller numbers of patients. These can be used at the phase I/II level of drug development to establish the probable efficacy/usefulness.

In rheumatic conditions where effective therapies have been demonstrated there is no a major problem with placebo use. This is a particular problem with studies of conventional drugs that require large patient numbers because of the insensitivity of the X-ray end points. Furthermore, it is only possible to show tests between active therapy and placebo rather than between two active therapies. The increased sensitivity and accuracy of the new imaging methods allow comparison between active comparators with the power to show differences with relatively small numbers. This has benefits for patients who are not exposed to placebo, for investigators who can compare current best therapies and new therapies, and for organisers of clinical studies with results produced much more rapidly.

There are number of biological markers that can be used for the assessment and follow-up of arthritis patients. The acute phase C-reactive protein is a very sensitive measure of inflammation and an excellent predictor of radiological damage. In biologic studies there is evidence of its predictive value very early in the follow-up process. There are a number of markers in serum and urine that can be used as surrogates of bone and cartilage damage. Their main use is when the normal predictors of damage are absent when early phase studies preclude the use of long-term follow-up.

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Biochemical markers of bone and cartilage in osteoarthritis

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Osteoarthritis (OA), the most prevalent joint disease, is characterized by abnormal and degraded cartilage, inflamed and/or thickened synovial tissue, and alterations of bone metabolism (sclerosis, cysts, osteophytes). The extracellular matrix of joint tissues is composed primarily of collagens including type I (bone and synovium), type II (cartilage) and type III (synovium) associated with proteoglycans (e.g. aggrecan in cartilage) and other glycoproteins. Potential biological markers for OA include matrix components (and/or their breakdown products), cytokines, and proteases [1].

Although bone turnover markers may reflect the focal abnormalities of subchondral bone metabolism in OA, circulating and urinary levels are more likely to reflect the overall skeletal turnover, which may be influenced by a variety of conditions including age, menopausal status, osteoporosis and other bone diseases. Major efforts have recently been made to develop more specific markers, especially those reflecting cartilage type II collagen synthesis (PIINP and PIICP) and destruction and synovial activity that plays a pivotal role in the pathogenesis of OA. Antibodies recognizing different type II collagen fragments have been developed and include those directed against the neo-epitopes generated by the collagenases, and against C-telopeptide breakdown fragments (CTX-II). We have characterized a pyridinoline derivative, glucosyl-galactosyl-pyridinoline (Glc-Gal-PYD), which is found in large amounts in human synovium and in very low levels in the cartilage and other soft tissues. Urinary Glc-Gal-PYD excretion has been found to be increased in knee OA and hip OA, and the levels show stronger correlations with symptoms of OA than do markers for bone and cartilage [2].

Recent prospective studies have shown that baseline levels of some cartilage markers are associated with a more rapid joint damage. In a study of 52 patients with knee OA, we found that low serum levels of PIIANP or high urinary CTX-II excretion were associated with faster joint destruction as evaluated over a 1-year period, either by plain radiographs or by arthroscopy [3]. Combining these two biological markers to obtain an index of uncoupling of type II collagen synthesis and breakdown was more effective in predicting cartilage destruction than measurements of a single marker. The predictive value of CTX-II has been confirmed in a larger study of volunteers followed prospectively for more than 6 years, where progression was assessed by joint space narrowing at the knees and hips [4]. In the ECHODIAH cohort, including 333 patients with hip OA followed prospectively for 3 years, we found that among a panel of 10 different biochemical markers of bone, cartilage and synovium activity, increased levels of urinary CTX-II and hyaluronic acid were strongly predictive of radiological progression independent of disease activity and radiological parameters [5]. One of the main issues that currently impair efficient development of structure OA modifying therapies is the low sensitivity of the plain X-ray, requiring long-term studies to show a significant difference between placebo-treated and active-drug-treated patients. In the COBRA study of patients with early rheumatoid arthritis, we found that the combined therapy (sulphasalazine + methotrexate + prednisone) produced a significant decrease in urinary CTX-II that can be observed within 3 months of therapy. The magnitude of the changes of CTX-II at 3 months were predictive – independent of changes in disease activity – of the changes in radiological scores after 5 years, indicating that the higher the decrease of CTX-II at 3 months, the lower will be the progression of joint damage at 5 years [6]. Such data are currently missing in OA, mainly because of the absence of treatments that have definitively been shown to reduce progression.

Recent work on biological markers for OA has made valuable progress; in particular, with the development of specific markers for the production and breakdown of type II collagen and of synovial membrane activity. Although, they are unlikely to be clinically useful for the identification of patients with OA, an increasing body of evidence suggests that combination of some biological markers will be useful adjuncts to radiography and magnetic resonance imaging to predict progression of OA. The rapid responsiveness of biological markers should prove valuable in the clinical development of drugs for preventing joint destruction and in monitoring their treatment effects in patients with OA.

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Markers in rheumatoid arthritis

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Proteinases play an important role in the pathogenesis of joint destruction, and matrix metalloproteinases (MMP) are believed to play a crucial role as they have the ability to degrade various compounds of the extracellular matrix of the joints, including collagen. The work described in this presentation focuses on the question of which MMPs and in what forms are they present in synovial fluid (SF) and the circulation of patients with joint pathology. Also, the applicability of the MMP measurements as markers of disease activity or joint damage progression is investigated. High levels of proMMP-3, proMMP-8 and proMMP-9 were found in SF and in the circulation of the rheumatoid arthritis (RA) patients as compared with the control population or osteoarthritis patients. Also MMP activity in MMP/α_2-macroglobulin (α_2M) complexes was shown to be higher in RA patients. Moreover, it was shown that proMMP-8 and proMMP-9 (collagenase 2 and gelatinase B) levels are related to the levels of collagen degradation products (hydroxyproline) in SF. Although there is only a trend towards correlation between proMMP-8 and hydroxyproline in SF, levels of proMMP-3 were not correlated with hydroxyproline levels. Comparison between systemic levels of inflammation (C-reactive protein) and MMP levels showed the opposite situation. Only proMMP-3 levels were correlated with C-reactive protein, and not proMMP-8 or proMMP-9 levels. These observations suggested that the aforementioned enzymes can be seen as candidate markers for the pathophysiological processes of joint inflammation and degradation. Moreover, MMP activity in MMP/α_2M complexes was considered a possible marker of the proteolytic system since increased levels of MMP activity were found in SF and in the serum of RA patients as compared with osteoarthritis patients. To investigate the predictive role of MMP levels in the circulation, we measured MMPs in patients in an Early Arthritis Clinic. The results show that proMMP-3 levels at the onset of the disease were predictive of cartilage loss at the end of the second year of the follow-up period. Moreover, proMMP-3 predicted joint damage progression independently from other known parameters such as sheared epitope or rheumatoid factor. Collagenase-2 (MMP-8) was not predictive of joint damage, and neither were high levels of proMMP-9. Based on these findings, we conclude that whereas MMP-8 and MMP-9 levels reflect the current status of the protheolytic system, MMP-3 levels at the onset of RA show the potential of the system to be destructive. It is extensively documented that protein levels of tissue inhibitor of metalloproteinase (TIMP)-1 in SF and in the circulation of RA patients are insufficient to counteract the increased production of MMPs. However, little is known about the activation of proMMPs *in vivo*. It was previously indicated that activated, but not TIMP-inhibited, MMPs can be neutralized by α_2M. We hypothesized that in pathological situations involving joint inflammation and destruction the high production of proMMPs leads to increased levels of activated MMPs, and because of the MMP/TIMP imbalance high levels of MMP/α_2M complexes may be found in these conditions. Thus, high levels of MMP/α_2M complexes would support the idea of a MMP/TIMP imbalance. To test this hypothesis, a method to measure MMP activity in MMP/α_2M complexes using small fluorogenic substrates, such as TNO211-F, was developed. Indeed, high levels of MMP in complexes with α_2M are found in SF and in the circulation of patients with joint pathology. The presence of increased levels of α_2M/MMP complexes shows that TIMP levels are insufficient to inhibit all activated MMPs; thus, the MMP/TIMP imbalance theory is supported by these findings. In conclusion, the results to be discussed show that high proMMP production leads to increased levels of activated MMP in SF and the systemic circulation of RA patients. Upon activation MMPs form complexes with α_2M in SF and in the systemic circulation of RA patients, which is at least partly responsible for neutralization of MMPs. MMP-1, MMP-3, MMP-8 and MMP-9 are likely to be involved the pathogenesis of RA as shown by the MMP profile in SF and the serum of RA patients. proMMP-3 levels at the onset of RA are predictive of the progression of joint damage. The MMP/TIMP imbalance is present in various joint pathologies. MMP/α_2M complexes are reduced in the systemic circulation of RA patients upon treatment with disease-modifying anti-rheumatic drugs.

Session VII – The targeting of autoantigens in systemic autoimmunity

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Antibody-initiated organ injury: apoptosis, inflammation and fibrosis in neonatal lupus

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Few diseases exemplify the integration of research from bench to bedside as well as neonatal lupus (NL). Although congenital heart block (CHB) and neonatal rash are strongly associated with maternal anti-Ro antibodies, the former occurs earlier and is permanent, and the latter is transient. Defining the pathogenicity of maternal antibodies in both manifestations must account for accessibility of the intracellular target antigen(s). *In vitro* studies suggest that one pathologic cascade leading to scarring of the conduction system may be initiated via apoptosis of the cardiocytes, resulting in translocation of Ro/La antigens and subsequent surface binding by maternal autoantibodies [1]. These opsonized cardiocytes are phagocytosed by macrophages, which secrete factors not only supporting an inflammatory response (secretion of tumor necrosis factor alpha [TNF-α]), but a fibrotic response (secretion of transforming growth factor beta [TGF-β]). The latter results in the transdifferentiation of fibroblasts into myofibroblasts, a scarring phenotype. *In vivo* studies support the molecular scenario identified in the co-culturing experiments. Based on immunohistochemistry of four fetal hearts identified *in utero* with CHB or isolated myocarditis, apoptosis was most extensive in fetuses dying early and was most pronounced in regions containing conduction tissue [2]. Deposition of IgG was observed in the CHB/myocarditis fetuses and colocalized to the apoptotic cells. Giant cells and macrophages (frequently seen proximal to IgG) were present in septal and thickened fibrous subendocardial regions, most apparent in the youngest fetuses. Septal tissue also revealed extensive areas of fibrosis and microcalcification in which a predominant smooth muscle actin-positive infiltrate (myofibroblast scarring phenotype) was observed [2]. TGF-β was expressed in septal regions and was present extracellularly in the fibrous matrix and intracellularly in macrophage infiltrates [3]. The detection of nuclear SMAD2 and PAI-1 provided strong evidence for TGF-β activation. Assessment of fetal genetic factors revealed that the TGF-β polymorphism Leu^{10} (associated with increased fibrosis) was significantly higher in CHB children (genotypic frequency, 60%; allelic frequency, 78%) than unaffected offspring (genotypic frequency, 29%, $P = 0.016$; allelic frequency, 56%, $P = 0.011$) [3]. With regard to NL rash, the immunohistology of biopsies taken from the lesional skin of three affected children revealed prominent TNF-α staining (cell-associated and extracellular) in the epidermal region and dermal–epidermal junction as well as the deeper fibroblast zone and adnexa [4]. The –308A allele (associated with higher production of TNF-α), HLA DRQB1*02, and HLA DRB1*03 were each present in the majority of children with rash (64%, 68%, and 64%, respectively). The frequency of all three 6p alleles together in one individual was significantly greater in the children with rash compared with children exposed to maternal anti-SSA/Ro antibodies who had either CHB or no manifestation of NL (59% versus 30%, $P = 0.02$) [4]. In summary, although maternal antibodies are common to both manifestations of NL, the genetic fetal

factor in rash appears to be maternally derived [4] and that in CHB paternally derived [3]. TNF-α may be one of several factors that amplify susceptibility, particularly in NL rash; however, the genetic studies, backed by the histological data, more convincingly link TGF-β to the pathogenesis of CHB. This profibrosing cytokine and its secretion/activation circuitry may provide a novel direction for evaluating fetal factors in the development of a robust animal model of CHB as well as therapeutic strategies in humans.

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Autoantibodies and cognitive impairment in systemic lupus erythematosus

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Many patients with systemic lupus erythematosus experience cognitive decline as their disease progresses. The pathogenic mechanisms include thrombosis, vasculitis, and drug toxicity. We have demonstrated that a subset of anti-DNA antibodies cross-reacts with *N*-methyl-D-aspartate NMDA receptors and can cause excitotoxic cell death. In mice with high serum titers of these antibodies, there is no evidence of brain damage until there is a breach in the blood–brain barrier. With a break in the blood–brain barrier induced by administration of lipopolysaccharide, the antibodies bind preferentially to hippocampal neurons that express *N*-methyl-D-aspartate receptors at high density. The antibodies mediate a noninflammatory apoptotic cell death. By 1 week following lipopolysaccharide administration, there is a 30% loss of hippocampal neurons. At 1 month there is no further loss of neurons, suggesting that the blood–brain barrier closes and the antibodies have no further access to brain tissue.

Mice experiencing hippocampal damage from antibody show decreased *N*-acetyaspartate in the hippocampus by magnetic resonance spectroscopy. An abnormal metabolism is detectable only in the hippocampus, confirming the selectivity of the damage. These mice also display impaired performance on tasks of memory function that are dependent on the integrity of the hippocampus. Thus, these studies provide a new model for cognitive impairment in systemic lupus and suggest that antibodies may mediate, through noninflammatory mechanisms, changes in cognitive function.

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Anti-DNA topoisomerase I autoantibodies bind directly to the cell surface of fibroblasts in patients with systemic sclerosis

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Objective Fibroblasts play a crucial role in the development of systemic sclerosis (SSc), and antifibroblast antibodies (AFAs) capable of inducing a proinflammatory phenotype in fibroblasts have been detected in SSc sera. In the present study, we examined the prevalence of AFAs in SSc and other diseases, and the possible correlation between AFAs and known antinuclear antibody specificities in SSc patients.

Methods Sera from 99 patients with SSc, from 123 patients with other autoimmune and nonautoimmune diseases, and from 30 age-matched and gender-matched healthy controls were examined. The AFA prevalence was assessed by flow cytometry and further characterized by indirect immunofluorescence, ELISA and immunoblotting. Anti-topoisomerase I (anti-topo I) from SSc sera was purified by affinity chromatography on topoisomerase I.

Results AFAs were more common in SSc patients (26%) than in any other diseases studied. The presence of AFAs was significantly associated with pulmonary involvement and death. SSc AFA-positive sera bound to all human and rodent fibroblasts tested but not to human primary endothelial or smooth muscle cells. All SSc AFAs strongly reacted with topoisomerase I by ELISA and immunoblotting. The binding intensity of SSc AFAs correlated strongly with reactivity against topoisomerase I on immunoblots of fibroblast extracts, and with the immunofluorescent pattern typical of anti-topo I on permeabilized cells. Total IgGs and affinity-purified anti-topo I from AFA-positive SSc sera were found to react with the surface of unpermeabilized fibroblasts by flow cytometry and by immunofluorescence and confocal microscopy.

Conclusion This is the first report establishing that AFAs in SSc are strongly correlated with anti-topo I and, furthermore, that anti-topo I itself displays AFA activity by reacting with determinants at the fibroblast surface.

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Mechanisms of autoimmunity in mice lacking the mer membrane tyrosine kinase

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There has been increased recent interest in the role of macrophages (Mp) and dendritic cells in systemic lupus erythematosus pathogenesis. The mer receptor tyrosine kinase, expressed on Mp and dendritic cells, mediates the binding of the former but not the latter to apoptotic cells. mer also is important in determining the cytokine profile that ensues after phagocytosis. Mice lacking mer develop antinuclear antibodies and rheumatoid factor. These autoantibodies appear to arise mostly from the splenic marginal zone. Stimulated spleen cells and peritoneal Mp from mer-deficient mice have increased expression of tumor necrosis factor alpha and IκBα, along with increased spontaneous expression of CD30L. The proinflammatory cytokine profile of mer-deficient mice may contribute to the immunogenicity of apoptotic debris. *In vitro* ligation of mer from normal Mp leads to diminished tumor necrosis factor alpha expression and increased IL-10 and IL-4. mer, which controls both phagocytosis and cytokine synthesis after exposure to apoptotic cells, may be an attractive target for therapeutic intervention in inflammatory and autoimmune disorders.

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Natural antibodies and autoantibodies bind to injured tissues, activate complement and cause organ damage

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Complement traditionally represents the first line of defense against infections. More recently, complement was shown to play a role in shaping the repertoire of the immune response. However, it is well established that uncontrolled and excessive complement activation significantly contributes to diverse pathologies ranging from inflammation, autoimmune tissue injury and injury that follows ischemia, hypoperfusion and reperfusion. In addressing the role of complement in tissue injury, two central questions arise: how is complement activated in each type of injury and to what extent is complement activation responsible for tissue injury? To obtain insight into these questions, we have adopted a mesenteric ischemia/reperfusion (IR) model of tissue injury because the local intestinal damage is associated with complement activation and recruitment and activation of neutrophils. Because immunoglobulin-deficient, *RAG-1*$^{-/-}$ mice are protected from IR injury and because some *Cr2*$^{-/-}$ mice have limited numbers of B1 B cells, we asked whether *Cr2*$^{-/-}$ mice have an altered response to IR injury. *Cr2*$^{-/-}$ mice are protected from IR injury that is completely restored after injection of normal IgG and IgM. In addition, antibodies with specificities against negatively charged phospholipids and DNA attach to injured tissue and activate complement, resulting in damage. We propose that certain autoantibodies, produced by B1 cells and constituting part of the natural antibody repertoire, bind to cryptic antigens expressed by injured cells and tissues, activate complement and effect tissue damage.

Session VIII – Is bone quality a factor for arthritic patients?

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Menopause and hormone replacement therapy: effects on the immune system, arthritis and bone

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Menopause is, among other things, associated with increased risk of bone loss and development of rheumatoid arthritis (RA). Numerous postmenopausal women in the Western world have replaced the endogenous estrogen by treatment with hormone replacement therapy (HRT) (i.e. estrogen + progestin) or estrogen replacement therapy (ERT) (i.e. estrogen alone). Until recently HRT was also regarded as preventive of cardiovascular disease, and possibly even dementia. However, recent findings in large placebo-controlled studies of long-term HRT and ERT in healthy postmenopausal women demonstrated an increased risk of coronary events, stroke, breast cancer and pulmonary embolism among the HRT-treated women. Consequently, today HRT is only recommended for 3–6 months in women with severe menopausal symptoms.

Is there any evidence that HRT has beneficial effects on established RA? We recently completed a 2-year controlled, randomized and prospective trial comparing calcium/vitamin D_3 + HRT and calcium/vitamin D_3 alone in patients with long-lasting RA. The outcome clearly showed that the HRT-treated patients responded with a significant decrease in Disease Activity Score 28 and laboratory signs of inflammation (sedimentation rate and hemoglobin), an increase in bone mineral density and less progression of radiographic joint destruction compared with controls [1]. Unfortunately, we do not know how HRT affects the increased risk for vascular disease in RA.

How could we develop a 'safe' HRT with preserved bone protective and anti-arthritic properties? It is an absolute necessity to increase our understanding of how estrogen exerts its effects in different biological compartments. We therefore conducted several studies in estrogen receptor (ER) knockout mice lacking one or both of ER-α and ER-β. In summary, these experiments show that ER-α is the dominant receptor for estrogen-mediated effects on immune organ development, T and B lymphopoiesis and inflammation, and that ER-β seems to play a regulatory role [2,3].

Another approach was to analyze the immune-modulating, antiinflammatory and anti-arthritic properties of a selective estrogen receptor modulator, raloxifene [4], and the synthetic compound 4-estren-3α,17β-diol (estren) [5]. Data from recent, as yet unpublished, experiments will be presented and discussed.

In conclusion, although certain side-effects of conventional HRT have been identified, there is good hope of finding HRT that reproduces only the beneficial effects of estrogen on bone and arthritis.

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Mechanisms of glucocorticoid induced osteoporosis

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Glucocorticoid-induced osteoporosis is the most common cause of secondary osteoporosis, and the degree of bone loss is related to the dose of glucocorticoids and the duration of the exposure. However, prolonged exposure to modest doses of glucocorticoids increases the risk of fractures and there is differential individual sensitivity to these steroids. Following treatment with glucocorticoids, there is an initial rapid loss of bone probably due to increased bone resorption and then a slower progressive loss probably secondary to decreased bone remodeling. Glucocorticoids decrease gastrointestinal calcium absorption and enhance bone resorption by increasing osteoclastogenesis since they stimulate the expression of receptor activator of NF-κB ligand and colony stimulating factor-1, and decrease the levels of osteoprotegerin, a soluble decoy receptor for receptor activator of NF-κB ligand. Glucocorticoids may prolong the life of mature osteoclasts under selected conditions. Eventually, glucocorticoids decrease bone remodeling by depleting the population of osteoblasts. This occurs by a decrease in osteoblastogenesis, and an increase in the apoptosis of mature osteoblasts and osteocytes. Glucocorticoids delay the maturation of immature stromal cells toward osteoblasts and inhibit the function of mature osteoblasts. Instead, glucocorticoids enhance adipogenesis, and this probably occurs at the expense of osteoblastic differentiation. The effect is secondary to the induction of CAAT enhancer binding protein beta and CAAT enhancer binding protein delta, and of peroxisome proliferator-activated receptor gamma, which inhibits osteoblastic differentiation. These effects suggest that glucocorticoids play a role in the trade of osteoblasts and adipocytes. This is confirmed further by the fact that Notch is induced by glucocorticoids and Notch inhibits osteoblastogenesis and enhances adipogenesis.

Clinically, the early effects of glucocorticoids on bone resorption can be reversed by the administration of bisphosphonates, whereas the inhibitory effects on bone formation might be reversed by parathyroid hormone. In conclusion, glucocorticoids have profound effects on skeletal cells that lead to the development of osteoporosis.

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Adverse effects of rheumatoid arthritis on bone remodeling

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Rheumatoid arthritis (RA) represents an excellent model for gaining insights into the adverse effects of inflammatory arthritis on local articular as well as generalized systemic bone remodeling. Bone loss manifested by focal erosions at the margins of diarthrodial joints represents the radiographic hallmark of RA. These lesions are produced by resorption of cortical bone at the bone–pannus junction. Inflammatory pannus can also extend into the marrow space, with accompanying subcortical and trabecular bone destruction. In animal models of inflammatory arthritis, erosion of subchondral bone contributes significantly to cartilage loss, as the scaffolding bone is destroyed by the inflammatory process. Preservation of subchondral bone integrity would be predicted to have a cartilage sparing effect even in the presence of continued intra-articular joint inflammation. Recent studies employing magnetic resonance imaging have shown that marginal joint erosions occur very early in the course of RA and progress throughout the disease [1,2]. The propensity of the inflamed pannus tissue in RA to induce bone resorption is probably related to its capacity to produce a variety of factors with potent osteoclast differentiation and activation activity. Particular attention has focused on receptor activator of NF-κB ligand (RANKL), a member of the tumor necrosis factor ligand family, because of the requirement of this factor for osteoclastogenesis. RANKL is expressed by synovial fibroblasts and activated T cells in RA synovial tissues [3]. In three different animal models of inflammatory arthritis, treatment with osteoprotegerin (the soluble receptor that inhibits RANKL activity) results in marked suppression of focal bone erosions [4–6]. In addition, mice possessing the null mutation for RANKL are protected from focal bone destruction in the serum transfer model of inflammatory arthritis [7]. These observations lend support to the concept that enhanced osteoclast-mediated bone resorption at the pannus–bone interface and in subchondral and trabecular bone play a critical role in the pathogenesis of focal articular bone erosions.

An additional observation in patients with active RA is the absence of bone repair radiographically. This finding suggests that the processes that regulate coupling of bone resorption and formation under physiologic conditions have been disrupted, and that the enhanced focal bone resorption associated with the synovial inflammatory lesion is not matched by a compensatory increase in bone formation. Of particular interest will be the determination of the effects of therapies that inhibit joint erosions on these focal bone remodeling events at the bone–pannus interface and in the subchondral bone.

In addition to the disordered focal bone remodeling associated with the synovitis, patients with RA also exhibit evidence of generalized axial and appendicular osteopenia at sites that are distant from inflamed joints [8]. The reduction in bone mass has been confirmed using multiple different techniques, and patients with RA have an increased risk of hip and vertebral fractures [9]. Assessment of biochemical markers of bone turnover indicates that there is a generalized increase in bone resorption, and that there is a correlation between disease activity and the rate of systemic bone resorption. Patients with greater disease activity exhibit enhanced rates of bone loss. It is likely that the disturbance in systemic bone remodeling is mediated by proinflammatory cytokines with osteoclastogenic activity that are released into the circulation from the inflamed joints. These factors probably then act systemically to produce a generalized increase in osteoclast-mediated bone resorption. Bisphosphonates have been shown to reverse systemic bone loss in patients with RA, but studies thus far have not shown that these treatment regimens reduce the progression of focal bone erosions [10]. It is likely, however, that new approaches for more effectively inhibiting osteoclast-mediated bone resorption will become available; for example, agents that specifically inhibit osteoclast formation or activity by targeting mediators such as RANKL. Whether preservation of the skeletal architecture, independent of, or in addition to suppression of joint and systemic inflammation, will impact on the progression of functional disability needs to be investigated in appropriately designed clinical trials.

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Subchondral bone architecture and quality in osteoarthritis

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Osteoarthritis (OA) is traditionally seen as a disease of articular cartilage. However, changes in the bone that have long formed part of the radiological diagnosis – subchondral sclerosis, cysts and osteophytosis – are increasingly recognised as being an integral part of the disease. It is curious that cysts, regions of bone loss, are found in sites that are loaded, whereas osteophytes grow at the joint margins in sites that might be expected to be relatively lightly loaded. This is contrary to normal expectations of mechanical regulation of bone formation and

resorption. Evidence from animal models of idiopathic OA suggests that bone changes may precede cartilage deterioration, which would indicate that they are part of the primary disease process. Patients with OA changes evident on hip radiographs were found to have a higher than average bone mineral density not only in the hip, but also in the distal radius, vertebrae and calcaneus.

These gross changes in the bone are reflected in the composition, structure and organisation of the trabecular bone and in the subchondral bone plate. There is massive proliferation of cancellous bone, by up to 60% by volume, throughout the femoral head and neck. Corresponding to this, histomorphometry has shown an increase in trabecular thickness, with a concomitant reduction in trabecular number, and an increase in osteoid width and eroded surfaces. A reduction in mineralisation is found in subchondral trabecular bone, perhaps reflecting a more rapidly formed woven bone-like tissue seen using scanning electron microscopy. This is reflected in a lower material density. Scanning electron microscopy also showed instances of abnormal bone formation in the trabecular spaces and a much greater number of Howship's lacunae indicating enhanced osteoclastic activity. A thickening of the subchondral bone plate has been reported in humans and cynomolgus macaques with OA, which also suggests an imbalance between bone formation and resorption. Changes are not limited to the affected joint. The iliac crest in women with hand OA has been found to have more bone, but with a mineralization profile shifting to higher densities, suggesting reduced bone turnover.

The proliferation of subchondral bone results in an increase in apparent, or tissue, modulus. Although the apparent modulus increases, the reduction in mineral content results in a reduction in the modulus of the bone matrix itself. Indentation studies showed a reduced hardness, and by implication modulus, in the subchondral bone matrix. Using thermal analysis and X-ray diffraction, we found no differences in crystallite size, unit cell dimensions or decomposition properties indicating, however, that the nature of the mineral itself, a carbonated apatite, was not altered.

Studies have implicated the receptor activator of NF-κB/receptor activator of NF-κB ligand/osteoprotegerin regulatory pathway by showing increased levels of mRNA for receptor activator of NF-κB and a decrease in the ratio of receptor activator of NF-κB ligand to osteoprotegerin. This ratio then failed to correlate with bone remodelling indices, unlike in the normal controls, suggesting a disruption of the regulation of bone remodelling. In addition, it has been suggested that the co-regulation of the mechanical properties of bone and cartilage does not function as normal. We have also shown that the femoral head contains twice the amount of fat per unit volume of cancellous bone tissue (including bone marrow) as osteoporotic bone and has elevated levels of (n-6) fatty acids, especially arachidonic acid.

Considerable changes in bone composition, architecture, quality and regulation are hallmarks of OA. It is still not clear whether these changes follow, precede or accompany the widely studied changes in articular cartilage. Subchondral bone does not appear to play a biomechanical role in the initiation of the disease. The widespread changes in bone, the frequent presence of multiple joint involvement and the link with obesity has lead to the suggestion of a systemic musculoskeletal disorder, in contrast to the traditional cartilage-first hypothesis.

Session IX – Pain in arthritis: the inside story

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Pharmacokinetic–pharmacodynamic modeling considering spinal and peripheral actions of nonsteroidal antiinflammatory drugs to optimize the treatment of inflammation-induced pain

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It has been documented that nonsteroidal antiinflammatory drugs (NSAIDs) exhibit pharmacological actions at both the peripheral and central levels. However, the actual participation of the mechanisms of action elicited at different anatomical sites after systemic NSAID administration is not clear. To gain further knowledge on this issue, the aim of the present work is to study the pharmacodynamics and pharmacokinetics of diclofenac, a NSAID prototype, using an integrative approach.

We have previously documented that local diclofenac administration at the site of injury induces antinociception in the formalin test, as well as in the pain-induced functional impairment model in the rat. This effect is reduced by nitric oxide (NO) and cyclic GMP synthesis inhibitors, as well as by potassium channel blockers [1,2]. We therefore assayed the effect of oral (systemic) diclofenac in the formalin test after pretreatment with either N^G-nitro-L-arginine Methyl ester (L-NAME), a NO synthesis blocker, or glibenclamide, a potassium channel blocker, given by two routes of administration: locally at the site of injury and intrathecally. L-NAME and glibenclamide given by these two routes significantly reduced oral diclofenac antinociception. These results suggest that, after systemic administration, effective diclofenac concentrations are achieved at the site of injury as well as at the spinal cord, and that in these two sites of action there is a participation of the L-arginine–NO–cyclic GMP–potassium channel pathway.

In a second series of experiments, diclofenac was administered locally at the site of injury (peripheral location), intrathecally, and simultaneously at the site of injury, intrathecally. Isobolographic analysis showed that there is an additive interaction between the effects at the peripheral and spinal levels. It is then likely that, after systemic administration, the observed antinociceptive effect is the result of the sum of peripheral and central mechanisms. Thus, effective diclofenac concentrations at central sites achieved after systemic administration probably are considerably lower that those required to observe an antinociceptive effect after direct injection, due to the interaction of mechanisms elicited at different anatomical locations. Therefore, from a pharmacokinetic point of view, the various central and peripheral sites of action can be considered as the affected compartment.

In a third experimental series, the pharmacokinetics and antinociceptive effect of diclofenac was assayed in the pain-induced functional impairment model in the rat, which allows a simultaneous determination of antinociception and blood drug concentration [3]. It appeared that there was no direct relationship between diclofenac in blood and antinociception. Antinociception, however, was significantly related to affect compartment concentrations estimated by pharmacokinetic–pharmacodynamic modeling, consistently with the earlier described pharmacodynamic results. Computer simulations using the derived pharmacokinetic–pharmacodynamic model showed that an oral formulation of diclofenac yielding a very fast absorption, and hence a high blood concentration peak, can optimize drug transfer to the affected compartment, resulting in a fast-onset, long-lasting antinociceptive response.

Our results show that the study of NSAID pharmacodynamics and pharmacokinetics using an integrative approach is useful for the characterization of the mechanisms of action involved after systemic drug administration, yielding information that allows the optimization of dosing regimens.

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Mechanisms of pain in arthritis

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During inflammation in a joint, patients experience hyperalgesia and sometimes resting pain. Hyperalgesia includes stronger pain upon

noxious stimulation (e.g. strong pressure or twisting the joint) and the experience of pain when stimuli are applied that are not felt painful under normal conditions (palpation, movements in the working range). Resting pain is felt without intentional stimulation. Neuronal mechanisms involved in arthritic pain are the peripheral sensitization (sensitization of primary afferent fibres supplying the joint) and central sensitization (sensitization of spinal cord neurons). The peripheral sensitization includes the sensitization of so-called polymodal nociceptors (high threshold receptors that are excited under normal conditions by noxious mechanical stimuli) and of silent nociceptors (neurons that are not excited even by noxious mechanical stimuli). When these nociceptors are sensitized in the process of inflammation they are rendered more excitable and then they even respond to normally non-painful stimuli. In addition, the enhanced input from sensitized nociceptors induces hyperexcitability of second-order neurons in the spinal cord. This central sensitization is an increased gain in the spinal nociceptive processing, and sensitized spinal cord neurons show stronger responses to stimulation of inflamed tissue but also to stimulation of adjacent and even remote healthy tissue. Thus the whole pain pathway is sensitized and this explains why, in the inflamed tissue, pain is evoked by stimuli that do not elicit pain under normal conditions [1].

Numerous mediators and ion channels are involved in the activation and sensitization of nociceptive neurons [1]. Importantly, some mediators that are involved in the pathophysiology of inflammation are also involved in the generation of peripheral and central sensitization (e.g. prostaglandins). Cyclooxygenases and prostaglandins in the periphery and in the spinal cord have been important topics in pain research [2]. During development of an acute inflammation in the knee joint, prostaglandin E_2 release in the spinal cord is significantly enhanced. This is in part due to an upregulation of cyclooxygenase-2 in the spinal cord that is already seen within 3 hours of inflammation [3]. The application of prostaglandin E_2 to the spinal cord produces hyperexcitability of spinal cord neurons similar to peripheral inflammation. When indomethacin is applied to the spinal cord before and during the development of joint inflammation, the development of central sensitization is significantly attenuated [4]. These findings show the importance of spinal prostaglandins in the generation of inflammation-evoked spinal hyperexcitability. However, the spinal administration of indomethacin to the spinal cord after establishment of inflammation did not reduce responses of spinal cord neurons to mechanical stimulation of the inflamed joint, raising the question of how important spinal prostaglandins are in the maintenance of spinal hyperexcitability [4]. Prostaglandin E_2 acts through EP1, EP2, EP3 and EP4 receptors. The generation of spinal hyperexcitability by spinal prostaglandin E_2 can be mimicked by spinal application of agonists at the EP1, EP2 and EP4 receptors. When the joint is inflamed and hyperexcitability is established, EP2 and EP4 receptor agonists fail to change responses of spinal cord neurons to mechanical stimulation of the joint. Interestingly, however, the spinal application of an agonist at the EP3 alpha receptor even reduced responses although it had no effect under normal conditions [5]. Thus considerable plasticity of EP receptor activation seems to determine the precise role of spinal prostaglandins in different phases of inflammation.

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Session X – Innate immunity and its relation to autoimmune diseases including rheumatic diseases

42

Innate immunity and Toll-like receptors

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A recently described family of receptors, called the Toll-like receptors (TLRs), may help explain what triggers the production of inflammatory mediators such as tumour necrosis factor and chemokines in diseases such as rheumatoid arthritis. TLRs recognize microbial products and trigger signalling pathways, which culminate in enhanced expression of immune and inflammatory genes. They are emerging as key players in the pathogenesis of infectious and inflammatory diseases. TLRs are defined by the presence of leucine-rich repeats and a signalling domain, which contains the Toll/IL-1 receptor (TIR) domain. An important finding is that the cystolic domain of the receptor for the cytokine IL-1 is similar to TLRs and that they signal in a similar way. Ten TLRs exist in humans and the best characterized are TLR4, which senses lipopolysaccharide from Gram-negative bacteria, and TLR3, which senses viral RNA. Signalling by each TLR is initiated by the receptor TIR domain and recruits via homotypic adapter proteins that contain TIR domains. Five such adapters have been discovered to date including MyD88, Mal (also known as TIRAP), Trif, Tram, and SARM. Signals activated include the transcription factor NF-κB and mitogen-activated protein kinases, which lead to induction of gene expression. Differences are emerging between TLRs in terms of which adapter is recruited by which TLR. This may lead to specificities in TLR signalling, with pathways being triggered that are specific for the elimination of the invading microbe.

Manipulation of proteins with TIR domains presents a number of opportunities for enhancing host defence or disease resistance, or strategies to limit inflammation. In the context of chronic inflammatory diseases, roles for TLRs in the pathogenesis of inflammation are emerging in conditions such as rheumatoid arthritis and systemic lupus erythematosus. Examples include the expression and action of TLRs in synoviocytes and a role for TLR9 in the production of rheumatoid factor from autoreactive B cells. Since TLRs are likely to be key drivers of TNF production, limiting their actions may have clinical benefit in inflammatory diseases. Additionally, since signals like NF-κB and p38 mitogen-activated protein kinase are activated by TLRs, inhibiting these processes will limit the effect of TLRs. Recent evidence, that certain receptor signals activated by TLRs may have an inflammatory rather than an immune role, raises the possibility of limiting the proinflammatory effects of TLRs without affecting roles in host defence. Furthermore, certain members of the TLR family (SIGIRR and ST2) may in fact be inhibitory receptors, and several negative signals generated during TLR action have been identified. As information on TLRs accumulates, more opportunities will present themselves for drug development.

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Regulation of B-cell tolerance by complement C4

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Systemic lupus erythematosus (SLE) is a B-cell-dependent autoimmune disease characterized by autoantibodies specific for nuclear antigens such as dsDNA and histones. Apoptotic blebs released from dying cells are thought to represent a major source of autoantigen. The cellular and molecular basis for SLE is not known but a major risk factor

is deficiency in products of natural immunity. For example, individuals deficient in serum complement proteins C1q or C4 almost always develop lupus. This presents a paradox as the complement system is known to mediate pathogenesis in SLE. In addition to its known role in inflammation, the complement system participates in humoral antibody responses via enhancement of B-lymphocyte activation, germinal center survival and maintenance of long-term effector responses.
Two nonmutually exclusive hypotheses for a protective role of complement are termed the clearance hypothesis and the B-cell tolerance hypothesis. The former proposes that complement (and natural immunity) protects by clearance of apoptotic blebs and sequesters them from the adaptive immune system. The tolerance hypothesis proposes that complement and natural immunity act in concert to localize lupus-antigens to sites within the primary and secondary lymphoid compartments where developing B cells undergo negative selection. To examine directly a role for complement C4 in protection from self-reactive B cells, we have bred the deficient mice with mice bearing rearranged immunoglobulin heavy chain and light chain anti-chromatin transgenes (Tg) (termed 564) specific for chromatin. On a normal C4-sufficient background, the self-reactive Tg B cells are blocked at the immature or transitional stage of development. By contrast, in the absence of C4, the self-reactive B cells continue to develop and enter the mature compartment. Moreover, they appear to secrete self-reactive antibody of both IgM and IgG isotypes. These results identify a functional role for C4 in regulation of self-reactive B cells and support the B-cell tolerance hypothesis.

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Complement and autoimmunity: new insights into old questions

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The innate immune system is now known to play an important role in modification of adaptive immunity. In addition, innate immunity is centrally important to the development of collagen-induced arthritis (CIA), a widely studied model for the human disease rheumatoid arthritis. Complement is a key component of the innate immune system, and complement activation in inflamed joints has been previously shown to both characterize active disease in patients with rheumatoid arthritis as well as to be essential to the development of CIA in mice and other similar animal models. There is little understanding, however, of the specific mechanisms by which complement influences cellular and humoral autoimmunity in CIA, or how complement activation fragments intersect with other innate or adaptive immune mechanisms in the disease process. We have recently found that the alternative complement pathway component factor B is centrally involved in disease pathogenesis in the CIA model. Following transfer of anti-type II collagen monoclonal antibodies into factor B-deficient mice, only modest inflammation results as compared with wild-type mice. In addition, expression of complement receptor 2 (CR2/CD21) and complement receptor 1 (CR1/CD35), molecules that are expressed on B lymphocytes and T lymphocytes as well as follicular dendritic cells and are known links for innate and adaptive immunity, is required to develop CIA. Specifically, elimination of CR2/CR1 expression by gene targeting results in a marked decrease in the generation of arthritis following immunization with type II collagen in DBA1/j mice. Of interest, despite substantial protection from disease in receptor-deficient mice, only modest changes occur in IgG anti-type II collagen antibody subclass and isotype responses. Understanding the mechanisms by which the alternative pathway as well as complement receptors modulate inflammatory arthritis should provide important insights into the relationships between innate and adaptive immunity.

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Insights into systemic lupus erythematosus pathogenesis through blood global gene expression analysis

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Systemic lupus erythematosus (SLE) is a prototype systemic autoimmune disease characterized by flares of high morbidity for which we have no predictors. We used oligonucleotide microarrays to analyze the genes expressed by blood mononuclear cells from active SLE patients, arthritis patients and healthy controls. Here we show that active SLE can be distinguished by a remarkably homogeneous gene expression pattern with overexpression of granulopoiesis-related and interferon (IFN)-induced genes. Using the most stringent statistical analysis (Bonferroni correction), 15 genes were found highly upregulated in SLE patients, 14 of which are either well-known or newly identified targets of IFN. The other gene is the defensin DEFA-3, a major product of immature granulocytes. A more liberal correction to the pairwise tests (Benjamini and Hochberg correction) yielded 16 additional genes, 12 of which are known to be IFN-regulated and four to be granulocyte specific. High-dose intravenous infusion of glucocorticoids, one of the standard treatments of disease flares, shuts down the IFN signature, further supporting the critical role of this cytokine in SLE. Furthermore, we have identified a set of 10 genes whose expression correlated with disease activity according to the SLE Disease Activity Index. The most striking correlation ($P < 0.001$, $r = 0.55$) was found with the formyl peptide receptor-like 1 protein that mediates chemotactic activities of defensins. Therefore, while the IFN signature confirms the central role of this cytokine in SLE, the microarray analysis of blood cells reveals that immature granulocytes may be involved in SLE pathogenesis.

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Regulation of innate and adaptive immunity and autoimmunity by Toll-like receptor 9

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Toll-like receptors (TLRs) are a family of immune defense proteins that appear to have evolved to detect molecules that are common to broad classes of pathogens, but are not present in our own cells. Specific TLRs can be activated with synthetic ligands to induce protective or therapeutic innate and adaptive immune responses. CpG-oligodeoxynucleotides (ODN) are TLR9 ligands that have proven particularly active for immunotherapy of cancer and infectious and allergic diseases in animal models. Several CpG-ODN are now in phase I/II human clinical trials for these indications, and have shown early evidence of efficacy, with excellent safety and tolerability. Patients in cancer clinical trials have been tolerating weekly injections of CpG-ODN for longer than 6 months. Objective disease responses have been documented in five different tumor types. A few subjects have developed positive anti-dsDNA ELISAs, but antinuclear antibodies generally have been negative and the synthetic TLR9 agonists in human trials have not induced autoimmune disease. Although stimulation of the TLR9 pathway does not appear to be *sufficient* for induction of autoimmune disease, this pathway does appear to be *required* for the full development of systemic autoimmunity in some mouse models, which suggests a therapeutic role for TLR9 antagonists. These results provide new insights into the mechanism of action for the anti-rheumatic disease activity of the anti-malarials. This class of compounds had been hypothesized to act by interfering with antigen processing and presentation, but these biologic activities only occur at concentrations that are approximately two orders of magnitude above the concentrations attained in treated patients. Surprisingly, the most potent biologic activity of the anti-malarials now appears to be their inhibition of TLR9 stimulation by immunostimulatory CpG motifs. Thus, TLR9 should be considered as a validated drug target, and the classic antimalarials as relatively weak TLR9 antagonists.

POSTER PRESENTATIONS

Session A

Inflammatory mediators: molecular mechanisms of joint destruction

47 (P1.1)

Molecular regulation of gene expression in chondrocytes by inflammatory mediators

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IL-1β, as the prototypic inflammatory cytokine, induces or activates a number of different transcription factors, including NF-κB, CCAAT/enhancer binding protein beta (C/EBPβ), C/EBPδ and certain Ets factors, which are involved in upregulating the expression of genes such as cyclooxygenase-2, matrix metalloproteinase (MMP)-13, and inducible nitric oxide synthase in chondrocytes and other cell types. IL-1β suppresses the expression of genes associated with the differentiated chondrocyte phenotype, including type II collagen (COL2A1) and aggrecan. We reported previously that IL-1β-induced Egr-1 inhibits COL2A1 promoter activity by binding to the −131/+125 bp core promoter and preventing the interactions among CREB binding protein, Sp1 and TATA-binding proteins [1]. This early response permits further transcriptional repression by IL-1β-induced factors that bind to upstream promoter sequences. The Ets factor ESE-1, which is epithelial-specific under physiological conditions, is expressed in rheumatoid arthritis and, to a variable extent, osteoarthritis synovial tissues. ESE-1 mRNA is induced by treatment of chondrocytes, synovial fibroblasts, osteoblasts, and monocyte-macrophages with IL-1β, tumor necrosis factor alpha, or lipopolysaccharide. After stimulation with IL-1β, ESE-1 mRNA appears within 30 min, peaks between 4 and 8 hours, and declines after 16 hours. This induction relies on the translocation of the NF-κB family members, p50 and p65 to the nucleus and transactivation of the ESE-1 promoter via a high-affinity NF-κB binding site [2]. Following induction, ESE-1 can directly activate IL-1-induced promoters by binding to two or more functional Ets sites [3].

To determine the mechanisms by which IL-1-induced transcription factors regulate COL2A1 gene expression, we used as a model the human chondrocyte cell line C-28/I2, in transient transfections with pGL2-COL2A1 constructs and as a source of nuclear factors for gel shift analysis. Our results indicate that ESE-1 acts as a direct repressor of COL2A1 promoter activity by binding to at least two of several Ets sites upstream of −131 bp and blocking protein–protein interactions between the SOX protein complex and the coactivator CREB binding protein/p300, thereby disrupting the basal transcriptional machinery. Egr-1, by displacement of Sp1, and NF-κB, as well as C/EBPβ and C/EBPδ [4], may also participate by direct or indirect mechanisms. These activities are consistent with early cytokine-activated events that are usually associated with gene activation but produce a negative response in the context of the COL2A1 promoter. The differential activation of upstream signaling events that result in induction of these transcription factors could explain the synergy and redundancy in cytokine responses. While ESE-1, by itself, is a potent negative regulator of COL2A1 promoter activity, IL-1β also stimulates the production of prostaglandin E_2 [5] and bone morphogenic protein-2 [6], both of which stimulate COL2A1 expression and may blunt the effects of the negative regulators. Thus, in the context of osteoarthritis cartilage, the initial events that activate chondrocyte synthetic activity probably result in activation of the normally inactive COL2A1 promoter, which would then be susceptible to transcriptional repression depending upon the balance of positive and negative regulators. Since IL-1β-induced transcription factors also upregulate genes associated with catabolic and inflammatory responses, including cyclooxygenase-2, MMP-13, and inducible nitric oxide synthase, and similar signaling pathways may be induced by adverse mechanical stress, the dissection of the molecular mechanisms involved may lead to the development of targeted therapies for blocking destruction of the cartilage matrix and promoting its repair.

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48 (P1.2)

Synergistic interactions of proinflammatory cytokines with oncostatin M: production of active collagenases and implications for joint destruction

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Oncostatin M (OSM) is a member of the IL-6 family that we previously showed could synergise with IL-1 to induce cartilage proteoglycan and collagen degradation in a cartilage explant culture system [1]; these observations now extend to IL-6 in the presence of its soluble receptor. A significant finding was the synergistic induction of the collagenase, matrix metalloproteinase (MMP)-1, which occurs via interplay between the janus-activated kinase/signal transducer and activator of transcription, activator protein 1 and mitogen-activated protein kinase pathways. Other collagenases such as MMP-8 and MMP-13 are also upregulated along with MMP-14 and MMP-3. This latter enzyme can activate the collagenases and an important feature of OSM may be its ability to activate enzymes that initiate the activation cascades that lead to the production of active collagenases. These studies have important implications for inflammatory joint disease since OSM (and indeed IL-6) have been proposed to be protective in rheumatoid arthritis. We also demonstrated that OSM can also exacerbate the effects of other important proinflammatory mediators such as tumour necrosis factor alpha (TNF-α) and IL-17.

We have continued molecular and cellular studies to discover the mechanism of action that leads to synergy. Using Affymetrix microarrays we have shown that a specific cohort of genes are upregulated by these cytokine mixtures that include MMPs, a disintegrin and metalloproteinases, activators, cell surface proteins and cytokines. Analysis using two-dimensional gel electrophoresis and proteomic analysis has confirmed that many of the corresponding proteins are made by chondrocytes after stimulation. Purification of specific proteins from conditioned culture medium has been undertaken to try and determine the specific collagenase responsible for collagen turnover.

In order to assess the effects of these cytokine combinations *in vivo*, we have assessed the effects of intra-articular gene transfer of OSM in

combination with either IL-1 or TNF-α on murine knee joints using recombinant adenovirus. Engineered adenoviruses were administered for only 7 days, after which time joints were fixed, decalcified and sectioned. Histological analyses indicated marked synovial hyperplasia and inflammatory cell infiltration for IL-1, TNF-α and OSM treated joints but not in controls (joints treated with an 'empty' adenovirus). The inflammation was more pronounced for both the OSM + IL-1 and OSM + TNF-α combinations with evidence of extensive cartilage and bone destruction. Significant loss of both proteoglycan and collagen was also seen for these combinations, and immunohistochemistry revealed an increased expression of MMPs with decreased tissue inhibitors of metalloproteinases in both articular cartilage and synovium. The effects of these combinations were significantly greater than those seen with any of the cytokines alone. Cytokine combinations also upregulated receptor activator of NF-κB/receptor activator of NF-κB ligand (RANK/RANKL) and increased the number of TRAP-positive cells. A significant increase in osteoclast formation and activation and bone damage was accompanied by marked upregulation of RANK/RANKL in inflammatory cells within the synovial tissue. Taken together, these data confirm that, *in vivo*, OSM can significantly exacerbate the effects of both IL-1 and TNF-α, resulting in inflammation and tissue destruction characteristic of that seen in rheumatoid arthritis. *In vitro* data showed that the damage to cartilage in the cartilage model system can be blocked by both transforming growth factor beta and insulin like growth factor-1.

These studies provide further evidence to implicate the upregulation of collagenases as a key factor in the destruction of collagen that occurs in joint pathology, and suggests that OSM is a potent mediator when found in the joint with other proinflammatory cytokines.

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49 (P1.3)

Endothelin-1 induces extracellular matrix degradation via matrix metalloproteinases induction in human osteosarcoma cells

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Extracellular matrix (ECM) degradation plays an important part in many musculoskeletal pathologies including arthritis and bone tumour. Matrix metalloproteases (MMPs) degrade the ECM and are thus important in the development of these pathologies. Furthermore, recent evidence suggest the existence of interactions between endothelin-1 (ET-1) and MMPs.

This study aimed at determining the effect of ET-1, Big ET-1 (the immediate precursor of ET-1) and IL-1β (a proinflammatory cytokine that induces MMP synthesis) on MMPs in order to determine the mechanism of action of these factors in chondrosarcoma and osteosarcoma cells. We have also studied the effects of the NF-κB inhibitor and the inhibitor of furine convertase that is involved in Big ET-1 maturation. Here we characterized the MMPs, ET-1 and ET-1 receptors in SW1353 and MG63 cells by western blot, zymography, northern blot, immunohistochemistry, RT-PCR, and *in situ* hybridization.

First, we showed that ET-1 and its two receptors (ET_A and ET_B) are constitutively expressed in osteosarcoma and chondrosarcoma cells. Then, we demonstrated that both ET-1 and Big ET-1 markedly induce synthesis and enzymatic activity of MMP-2 and that enzymatic activity is significantly increased when compared with MMP-9. Furthermore, inhibition of NF-κB activation (by pyrrolidinecarbodithioic acid) blocked MMP-2 production and activity, indicating the involvement of NF-κB. Similarly, inhibition of Big ET-1 maturation by the furin convertase inhibitor abrogated MMP-2 synthesis.

ET-1 and as its immediate precursor Big ET-1 can be considered as autocrine factors contributing to the activation of MMPs, thus favouring ECM degradation. MMPs, particularly MMP-9 and MMP-2, are hyperexpressed in chondrosarcoma and osteosarcoma cells. Their production is stimulated by ET-1, Big ET-1 and IL-1β while it is diminished when the NF-κB and furine convertase pathways are inhibited. Our study provides new insights on the catabolic role of ET-1 in ECM degradation. Thus ET-1 could be involved in diseases where ECM destruction is caused by an excess of MMP, including bone tumour, degenerative and inflammatory articular diseases. In an attempt to diminish this degradation, it would be beneficial to propose a double inhibition of both ET-1 receptors and MMPs.

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50 (P1.4)

Endothelin-1 in osteoarthritic chondrocytes triggers nitric oxide production and upregulates collagenase production

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Background Cartilage degradation in osteoarthritis (OA) and rheumatoid arthritis constitutes a major structural change in the joint, which may severely impair its function causing pain and disability. This degradation is accompanied by the release in the synovial fluid of degraded matrix constituents that primarily result from an increased matrix catabolism. Various factors are directly involved in this process. Endothelin-1 (ET-1), a potent vasoconstrictor and pro-mitogen peptide for many cell types, including chondrocytes, was recently identified as one such factor.

Objective We previously demonstrated that ET-1 induces matrix metalloproteinase (MMP)-1 and MMP-13 synthesis, secretion and activation. Here, we investigated the mechanism by which ET-1 induces the production of these two MMPs.

Methods Human OA chondrocytes were cultured in the presence of ET-1 with or without inhibitors of protein kinase or LY83583 (an inhibitor of soluble guanylate cyclase and of cGMP) and then MMP-1, MMP-13 and nitric oxide (NO) levels were measured by ELISA and Griess reaction, respectively. Additionally, inducible nitric oxide synthase (iNOS) and activated forms of p38 mitogen-activated protein kinase, p44/42, SAP/JNK and serine threonine kinase Akt were determined by western blot, Bad and Bcl2 proteins by immunocytochemistry and apoptosis by TUNEL.

Results ET-1 greatly increased MMP-1 and MMP-13 production, NO release and iNOS expression. LY83583 decreased the production of both MMPs below basal levels, whereas the inhibitor of p38 kinase, SB202190, suppressed ET-1-stimulated production only. Similarly, the ET-1-induced NO production was partially suppressed by the p38 kinase inhibitor and completely suppressed by the PKA kinase inhibitor, KT5720, and LY83583, suggesting the involvement of these enzymes in ET-1 signaling pathways. ET-1 does not induce apoptosis and could even have a protective effect through the induction of Akt phosphorylation.

Conclusions In human OA chondrocytes, ET-1 controls the production of MMP-1 and MMP-13. ET-1 also induces NO release via iNOS induction. Thus, ET-1 and NO should become important target molecules for future therapies aimed at stopping cartilage destruction.

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51 (P1.5)

Intracellular IL-1Ra exhibits unique antiinflammatory properties

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IL-1Ra exists in four isoforms, three of which are created by alternate transcriptional splice mechanisms and remain in the cytoplasm. The objective of these studies was to determine whether the major intracellular isoform of IL-1Ra, the 18 kDa type (icIL-1Ra1), bound specifically to cytoplasmic proteins and exerted antiinflammatory effects inside cells. A yeast two-hybrid screen with HeLa cell lysates indicated binding of icIL-1Ra1 to the third component of the COP9 signalosome complex (CSN3). The CSN complex contains eight subunits, is found in the cytoplasm and nucleus of all mammalian cells, and appears to function as an interface between signal transduction pathways and ubiquitin-dependent proteolysis [1]. The binding of icIL-1Ra1 to CSN3 was confirmed by far western blot analysis and sedimentation in a glycerol gradient; icIL-1Ra1 bound only to CSN3 and not to the other seven components of the CSN. Glutathione pull-down experiments showed that only icIL-1Ra1, and not the other isoforms of IL-1Ra, bound to CSN. Co-immunoprecipitation experiments also indicated binding only of icIL-1Ra1 to the CSN. In addition to binding specifically to CSN3, icIL-1Ra1 inhibited phosphorylation of IκB, p53 and c-Jun by CSN-associated kinases. Furthermore, icIL-1Ra1 blocked p53 phosphorylation by recombinant protein kinase 2 and protein kinase D, two kinases associated with the CSN.

To examine the biological function of icIL-1Ra1, this cDNA was transfected into the intestinal epithelial cell line Caco-2, which expresses no endogenous IL-1Ra isoforms. The transfected icIL-1Ra1 inhibited IL-1-induced IL-6 and IL-8 production; this effect was not due to release of icIL-1Ra1 by the cells and blockade of IL-1 receptors [2]. Furthermore, the levels of IL-1-induced IL-6 and IL-8 production in various keratinocyte cell lines were inversely related to the baseline levels of icIL-1Ra1 in the cells. The keratinocyte line A431 contained large amounts of icIL-1Ra1 and exhibited weak production of IL-6 and IL-8 after culture with IL-1. In contrast, KB cells contained no icIL-1Ra1 and exhibited a robust production of IL-6 and IL-8 after stimulation with IL-1. Transfection of icIL-1Ra1 into KB cells led to inhibition of IL-1-induced IL-6 and IL-8 production. To explore whether these antiinflammatory effects of icL-1Ra1 are mediated through interaction with CSN3, experiments are in progress to examine IL-1-induced cytokine production in keratinocyte cell lines after blockade of CSN3 or icIL-1Ra1 production using specific siRNAs. Similar experiments on the effects of icIL-1Ra1/CSN3 interactions on cytokine and matrix metalloproteinase production in human synovial fibroblasts are also in progress.

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52 (P1.6)

Oral treatment with PD-0200347, an $\alpha_2\delta$ ligand, reduces the development of experimental osteoarthritis by inhibiting the chondrocytes metalloproteases and inducible nitric oxide synthase gene expression and synthesis

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The aim of this study was to examine the *in vivo* effects of PD-0200347, an $\alpha_2\delta$ ligand of voltage-activated Ca^{2+} channels and a member of the gabapentin family, on the development of cartilage structural changes in an experimental osteoarthritis (OA) dog model. We examined its effects on the major pathways involved in OA cartilage degradation including metalloproteases (MMPs), the inducible form of nitric oxide synthase (iNOS) and IL-1β.

OA was surgically induced in dogs by sectioning the anterior cruciate ligament. OA dogs were divided into three groups and treated orally with placebo, with 15 mg/kg/day PD-0200347, or with 90 mg/kg/day PD-0200347. Dogs were sacrificed 12 weeks after surgery. The severity of lesions was scored macroscopically and histologically. Cartilage specimens from femoral condyles and tibial plateaus were processed for quantitative PCR and immunohistochemistry. Specific probes and antibodies were used to study IL-1β, iNOS, MMP-1, MMP-3 and MMP-13 mRNA and protein levels, respectively.

No clinical signs of drug toxicity were noted in the treated animals. PD-0200347 treatment at both dosages tested (15 or 90 mg/kg/day) reduced the development of cartilage lesions. There was a reduction in the score of lesions, and statistical significance ($P < 0.01$) was reached at the highest dosage of the drug. This score reduction was mainly related to the decrease in the lesion surface size. Quantitative PCR and immunohistochemical analyses showed that PD-0200347 treatment also significantly reduced key OA mediators, IL-1β, iNOS, MMP-1, MMP-3 and MMP-13 gene expression and synthesis.

This study demonstrated the efficacy of PD-0200347 at reducing the progression of cartilage structural changes in an OA dog model. It also showed that this effect is linked to the inhibition, at the transcriptional level, of the major pathophysiological mediators. The mechanism of action could be related to a significant reduction of Ca^{2+} current amplitude via voltage-gated calcium channels, which in turn downregulates the downstream signaling pathways.

Assessment of clinical and structural changes of arthritis

53 (P2.1)

Nonsteroidal antiinflammatory drug use may be protective to cartilage in osteoarthritis of the hip and knee

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Purpose Nonsteroidal antiinflammatory drugs (NSAIDs) are used to alleviate osteoarthritis (OA) symptoms and are not considered disease-modifying. We studied NSAID use in subjects with hip or knee OA to investigate the relationship between NSAIDs and OA severity.

Methods Subjects (seen over 2 years at teaching hospitals in London, Ontario) were referred to orthopedic surgeons or rheumatologists, had radiographs available, but had no inflammatory arthritis or evidence of secondary OA. Subjects were divided into mild, moderate (mod) and severe OA by X-ray (worst joint selected). A questionnaire was mailed asking about current/ever use of specific NSAIDs (by generic and trade names) including over the counter and acetylsalicylic acid. Coxibs were not available over the study period. A toal of 608 subjects

were studied, having mild (71), mod (129) and severe (408) OA, with a disease duration of 10 years in each group (62% were women, who had proportionately more mild disease than men).

Results Those with severe versus mild OA were older (70 versus 61 years), so age was adjusted for. There was an inverse dose–response with use of > 3 NSAIDs being used by mild versus severe OA (odds ratio 3.7), mild versus mod OA (odds ratio 2.5) and mod versus severe OA (odds ratio 1.5) ($P < 0.0001$), and the same was found with > 2 NSAIDs ($P < 0.0002$); and 7% of mild, 15% of mod and 12% of severe OA had received no NSAIDs ever. Only one NSAID (ever used) was increased in severe OA (sulindac, $P < 0.008$) compared with naproxen, tiaprofenic acid and diclofenac, more frequently used in mild than mod or severe OA ($P < 0.01$). Other NSAID use ever was not different between the groups (including indomethacin, thought to be chondrodestructive), but numbers using indomethacin were small. Limitations may include inability to study accurately the duration of NSAID use, older age in those with severe OA (which could affect NSAID treatment choice), recall bias and unknown confounders. We did not illicit body mass index or pain level in each group.

There may be biases for referring to different specialists as the rheumatologists prescribed NSAIDs more often and surgeons treated more subjects with severe OA ($P < 0.0001$). When analyses were stratified by specialist type, there were no differences between mild, mod and severe OA for NSAID use in subjects treated by rheumatologists; however, the trend for increased NSAID use with milder OA was evident in those seen by orthopedic surgeons. When stratified by knee and hip OA, the dose–response remained ($P < 0.003$ and $P < 0.03$, respectively). Subjects were followed by general practitioners, so we assumed past NSAIDs should have been prescribed equally in all groups, but this was not the case.

Conclusions Those with more severe OA are more rapidly progressive (having similar disease duration to the other groups) and could be innate NSAID nonresponders. However, one would assume that they should be exposed to more NSAIDs (looking for one to be helpful). We conclude that NSAID use and the number of NSAIDs used was greater in mild radiographic OA and postulate that lowering prostaglandins in the joint with NSAIDs may be protective for cartilage. Prospective studies are needed to confirm observations. Some *in vitro* models of NSAIDs in OA support our observations.

54 (P2.2)

Chondral defects: genetic contribution and relevance and associations with pain, age, body mass index, joint surface area, cartilage volume and radiographic features of osteoarthritis

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The etiology and significance of nonfull thickness chondral defects is uncertain. This study had two objectives. Firstly, to describe the differences in chondral defect prevalence and severity between offspring of those with severe knee osteoarthritis (OA) and age-matched and sex-matched controls without this history and, secondly, using this population as a convenience sample, to describe the associations between chondral defects and pain, age, body mass index, joint surface area, cartilage volume and radiographic features of OA. We interviewed 372 male and female adults aged between 26 and 61 years (58% female, mean age 45 years). Of these, 50% had a parent who has severe knee OA requiring knee replacement and 50% were age-matched and sex-matched randomly selected controls. Chondral defects, cartilage volume and joint surface area was determined at the patella, medial and lateral tibial compartments of the right knee by quantitative magnetic resonance imaging. Anthropometric factors were measured while knee injury history, knee pain and occupation involving significant bending were assessed by questionnaire. Radiographic knee OA was assessed by semi-flexed AP views. Chondral defects were surprisingly common and were more prevalent and severe in the offspring (57% versus 43%, $P = 0.007$; defect score 5.7 versus 5.1, $P = 0.002$). This was independent of other factors with the exception of pain and joint surface area, suggesting these three factors are all linked. In addition, chondral defects at the patella and femoral but not tibial sites were associated with knee pain but not injury history. Defect prevalence and severity also increased with increasing age and body mass index, especially in females. Adjustment for radiographic features of OA decreased the magnitude of these associations, suggesting they are directly relevant to OA. Defect prevalence and severity also increased with osteophytes and increasing joint surface area and were associated with decreased joint space width and cartilage volume. Finally, knee cartilage defect severity was associated with a urinary marker of cartilage breakdown ($r = +0.18$, $P < 0.001$). In conclusion, knee cartilage defects are common, have a genetic component that is linked to the genetic contribution to knee pain and bone size, and may have a role in the genetic pathogenesis of knee OA. Furthermore, the associations between defects and OA risk factors, joint surface area and cartilage breakdown suggest that nonfull thickness defects in younger life may be a marker of OA risk in later life.

55 (P2.3)

Quantitative measurements of articular cartilage by magnetic resonance imaging

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There is now ever-increasing acceptance that magnetic resonance imaging (MRI) can provide valuable, possibly unique, measurement data for an articular joint as a complete organ. Thus a single MRI scan, duration 10 min, which can be measured with most whole body scanners, visualises not only all the soft tissues within the joint capsule (articular cartilage, ligaments, menisci) and those surrounding it (muscles, tendons, blood vessels), but also the bone (both cortical and trabecular). Importantly, recent software developments enable quantitation of the dimensions of those structural elements. Particular attention has focused on human knee articular cartilage and a large number of studies have already reported measurement of total volumes or local volumes, and of mean cartilage thickness for an entire condyle or more localised regions.

This poster will provide an overview of studies from the Herchel Smith Laboratory that demonstrate the practical realities of quantitative measurements relevant to the longitudinal studies that must be made for human drug trials. The specific questions that we have explored since 1995 include the following:

- Is it best to make 'focal' or 'total' measurements?
- How to enhance reproducibility of spatial relocation for serial measurements?
- What are the best statistical definitions of measurement repeatability?
- How to decrease interobserver variations of spatial measurements?
- How to enhance measurement ergonomics?
- How to automate the measurement processes?

Acknowledgement Herchel Smith endowment.

56 (P2.4)

Elevated C-reactive protein levels in osteoarthritis are associated with local joint inflammation

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Purpose Previous studies have demonstrated an association between osteoarthritis (OA) progression and inflammation as measured by systemic C-reactive protein (CRP) levels. We hypothesized that CRP levels in patients with OA are pathogenically linked to synovial membrane inflammation. This study investigated the relationship between CRP and local synovial appearance, synovial histology, and synovial fluid IL-6 levels.

Methods Patients with idiopathic OA undergoing either total hip or knee arthroplasty or knee arthroscopic debridement were identified. Synovial membrane inflammation was graded intraoperatively and by

histologic examination of samples obtained from standardized locations. Synovial fluid IL-6 levels and plasma CRP levels were measured using a high-sensitivity ELISA.

Results Twelve patients with early OA and 37 patients with end-stage OA were identified. These OA patients were dichotomized into two groups: high CRP (CRP level > 3 μg/ml) and low CRP (CRP < 3 μg/ml). The high CRP group demonstrated elevated synovial fluid IL-6 levels ($P < 0.001$) as well as increased intraoperative and histologic synovitis scores ($P = 0.045$ and $P = 0.03$, respectively) compared with the low CRP group. There was a highly statistically significant correlation between synovial fluid IL-6 levels and systemic CRP levels (rho = 0.66, $P < 0.001$).

Conclusions We have demonstrated an association between a marker of systemic inflammation and local synovial inflammation in patients with OA. The more aggressive disease seen in OA patients with elevated CRP levels may be mechanistically linked to a more inflammatory synovial response in the diseased joint.

57 (P2.5)

Influence of corticosteroids on C-reactive protein in patients with rheumatoid arthritis

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Background C-reactive protein (CRP) is an acute phase reactant. It is usually increased in rheumatoid arthritis (RA) patients when disease is most active. These patients would be expected to participate in clinical trials, high CRP being a frequent requirement for inclusion. At the same time this group of patients is usually treated with moderate to high dose steroids. Steroids have the potential to decrease CRP without modifying disease progression. It has been our clinical observation that very active patients are probably not influenced very much by steroids, but patients with moderate disease activity who use low doses of steroids have their CRP lowered or suppressed.

Hypothesis Patients treated with low doses of corticosteroids would have a lower CRP than those treated with no steroids, precluding them from participating in clinical trials.

Methods We studied the correlation between CRP and corticosteroid doses in RA patients, fulfilling the American Rheumatism Association 1987 criteria, treated with oral steroids, disease-modifying antirheumatic drugs, and cyclooxygenase-2 selective inhibitors. Patients were on stable doses of their medications. CRP and steroids were measured during 250 visits performed in 65 patients. Patients were divided into three groups: those with ≤ 5 mg prednisone per day, those with > 5 mg prednisone per day and those without steroids. All other drugs remained stable.

Results Patients on ≤ 5 mg/day prednisone showed a significantly lower CRP compared with the patients not using steroids (85.71 versus 59.09, $P < 0.001$). However, patients with > 5 mg prednisone per day had a significantly higher CRP compared with patients with ≤ 5 mg/day prednisone (91.98 versus 59.09, $P < 0.001$).

Conclusion Randomized clinical trials are the current method to evaluate new therapies in RA. Patients are selected by strict inclusion and exclusion criteria including high CRP. Severe RA patients with very high CRP treated with more than 5 mg prednisone are not influenced by steroids, but moderately active patients on more than 0 but ≤ 5 mg/day prednisone could be excluded from clinical trials as a consequence of lowered CRP. A prospective, randomized, clinical trial is underway to prove or disprove this hypothesis.

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Clinical and laboratory aspects of immune diseases

58 (P3.1)

Immune tolerance: DNA vaccine as strategy in autoimmune diseases

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Autoimmune diseases (AID) affect 5–8% of occidental populations. Theses AID are characterized by the destruction of self-tissue following activation of autoimmune responses mediated by T cells or B cells, or both. Manipulations of immune responses to protect against autoimmunity as potential immunotherapeutic implications using DNA vaccination could help to treat and/or prevent AID.

Recently, we have generated an animal model to study the role of the molecular mimicry in breaking tolerance to a neo-antigen in the liver. Transgenic (Tg) mice expressing the nucleoprotein (NP) gene from lymphocytic choriomingitis virus (LCMV) as neo-self antigen in their hepatocytes develop an autoimmune hepatitis after immunization with plasmids coding for intracellular or secreted LCMV-NP forms [1]. Four groups of transgenic mice (Tg-NP) were immunized via the intramuscular route with both these plasmids (pNP) alone or co-injected with IL-12-expressing plasmid (pIL-12). NP-specific serum antibody titres were detected in an ELISA test.

All animals injected with different plasmid-based gene expression vectors showed different anti-NP antibody profiles. The IgG_1 subclass is an indication of Th2 immune response, whereas IgG_2 subclass production reflects a Th1 immune response. Mice vaccinated with intracellular form-coding plasmid alone or with pIL-12 developed an antibody response characterized by more pronounced Th2 profiles (IgG_1 isotype). In contrast, those injected with the secreted form injections alone or with pIL-12 produced exclusively IgG_2 isotypes indicative of a Th1 response. Theses results suggest that immune response profiles provoked by DNA vaccine seem to be dependent on the protein form rather than on the cytokine adjuvant or on the administration route of the DNA vaccine. Even if cytokine (IL-12) driving B cells and T cells to a Th1 phenotype response was added, specific serum antibody titres were significantly higher in mice co-injected with the intracellular form compared with those co-immunized with the secreted form.

The form of autoantigen used in DNA vaccination may induce protection from autoimmunity by inducing a cytokine shift in the immune response to the antigens responsible for the disease. Moreover, the induction of shift strategies could orient the profile of T cells responding to disease target antigens from Th1 to Th2 or Th2 to Th1 according to the autoimmune process involved in each AID in order to induce a selective protective immune response.

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59 (P3.3)

Protective role of interferon gamma in murine antigen-induced arthritis

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Objective In human rheumatoid arthritis interactions of immune cells with resident cells, especially synovial fibroblasts, play a crucial role in the perpetuation of inflammatory and destructive processes in affected joints. Apart from macrophages, Th cells are predominant in cell infiltrates, and Th cell responses are specified as Th1 type. As interferon gamma (IFN-γ)-secreting Th1 cells are suspected to promote autoimmune diseases, there is the notion that a shift of a Th1 response towards a Th2 response might ameliorate arthritis. However, in different models and therapy studies conflicting results are described. Thus, we investigated the role of IFN-γ in murine antigen-induced arthritis (AIA), a model with homologies to human rheumatoid arthritis in terms of histopathology, chronicity and responses to several immunomodulatory drugs.
Methods AIA was evaluated in IFN-$\gamma^{-/-}$ mice in comparison with C57BL/6 wild-type mice by clinical, histological and immunological parameters. Furthermore, Th cells isolated from immunised mice were separated according to their IFN-γ on the cell surface into IFN-γ^{+} and IFN-γ^{-} Th cells and than transferred into naive mice, which received at the same time a single injection of antigen (methylated bovine serum albumin) into the knee joint for arthritis induction. In cocultures with synovial fibroblasts, the stimulatory capacity of IFN-γ^{-} and IFN-γ^{+} Th cells was tested *in vitro*, analysing the levels of matrix metalloproteases, IL-6, and nitric oxide in supernatants.
Results IFN-$\gamma^{-/-}$ mice developed a more severe arthritis than wild-type mice with higher knee joint swelling, stronger acute inflammation and increased chronic joint destruction, even though IgG levels (total and methylated bovine serum albumin-specific) were reduced. Application of rIFN-γ ameliorates the severity of AIA in IFN-$\gamma^{-/-}$ mice. Congruently, the transfer of IFN-γ^{-} Th cells from immunised wild-type mice into naive mice led to a more severe arthritis than the transfer of IFN-γ^{+} Th cells. Furthermore, IFN-γ^{-} Th cells had a stronger stimulatory capacity for matrix metalloproteases and IL-6 in synovial fibroblasts *in vitro*, whereas IFN-γ^{+} Th cells were more potent nitric oxide inducers. Inducing processes are mainly driven by direct cell contact.
Conclusion These experiments suggest a protective role of IFN-γ in the experimental arthritis model. The lack of IFN-γ in Th cells exerted a higher disease-promoting effect *in vivo* as well as a higher stimulatory capacitiy to synovial fibroblasts *in vitro*. The recent data generated in experimental models indicate a more complex role of IFN-γ in the pathogenesis of Th1-mediated autoimmune diseases than originally proposed.

60 (P3.4)

Regulatory role of C5a anaphylatoxin on the FcγRs effector system in immune complex-induced diseases

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Enhanced effector cell activation to deposited autoantibodies and immune complexes (IC) is a significant factor causing inflammatory responses in immunologic diseases like autoimmune hemolytic anemia, rheumatoid arthritis, lupus nephritis and Goodpasture`s syndrome. Recent studies in animal models show that the development of such diseases is caused by the impairment of balance between activating/inhibitory Fc receptors (FcγRs). The ratio of the opposing FcγRs is critical in determining whether an antibody-dependent response will result. In the present study we aimed to investigate the role of C5a anaphylatoxin on the FcγR effector system in IC lung injury.
We analysed the reverse passive arthus reaction in the lungs of mice lacking different FcγRs, or C5aR. The expression of FcγR mRNA/protein on alveolar macrophages (AM) effector cells was studied by TaqMan real-time PCR and FACS analysis. The contribution of AM to the IC alveolitis was investigated using the AM-depletion/AM-transfer technique. The autocrine synthesis of C5a by AM and the role of G-protein-dependent signalling was studied using *in vitro* stimulation of MH-S AM cells with IgG_1 IC.
We have shown that C5a anaphylatoxin is produced in the lung of mice already 2 hours after IC deposition. Moreover, C5a:C5aR interaction exacerbates IC inflammation by altering the ratio of activating to inhibitory FcγR expression on AM, enhancing the former and suppressing the latter. Elimination of AM results in diminished IC alveolitis similar to that observed in FcγRIII$^{-/-}$ and C5aR$^{-/-}$ mice, as well as in AM-deficient mice with transplanted FcγRIII$^{-/-}$ or C5aR$^{-/-}$ AM. *In vitro* blockade of C5aR on MH-S cells using anti-C5aR antibodies, or pertussis toxin, confirms IgG IC-dependent generation of C5a by AM.
These data establish the critical link between complement and FcγRs in the initiation of IC lung injury. Moreover, AM effector cells provide cellular cross-talk of C5a and FcγR effector systems. *In vitro* studies suggest the central role of G-protein-dependent C5aR signalling in the control of FcγR activation of AM.
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61 (P3.5)

Differential modulation of cysteinyl leukotriene receptor 1 and 2 expression by distinct Toll-like receptor agonists in monocyte-derived dendritic cells

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Background Dendritic cells (DC) acquire, during their maturation, the expression of the chemokine receptor CCR7 and the ability to migrate to lymph nodes in response to MIP3 beta (CCL19). This migration is impaired in mice lacking the leukotriene (LT) C_4 transporter and is restored by addition of exogenous LTC_4.
Objectives To further define the role of LT in human DC function, we studied their expression of LT receptors during their differentiation from monocytes and their maturation.
Results Monocyte-derived immature DC had a lower expression of the LTD_4 receptor (CysLT1) than monocytes, whereas their expression of the LTC_4/LTD_4 (CysLT2) and the LTB_4 (BLT1) receptors remained stable. Maturation of DC with lipopolysaccharide, a classical Toll-like receptor (TLR)4 agonist, reduced CysLT1 expression by a further 50%, whereas CysLT2 expression was increased and BLT1 expression was only marginally reduced. Zymosan, a TLR2 agonist, reduced all receptor levels, whereas the TLR3 agonist poly I:C had no effect on CysLT expression. LTC_4, more than LTD_4, was able to enhance human DC migration in response to CCL19 and promote DC activation of T-cell proliferation, when DC were matured with lipopolysaccharide or zymosan, but not polyI:C.
Conclusion Our data suggest that human DC may differentially respond to LT, depending on their maturational stimuli, and thus potentially affect both innate and adaptive immunity in the context of inflammation.
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62 (P3.6)

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

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The receptor activator of NF-κB/receptor activator of NF-κB ligand (RANK/RANKL) pathway is critical in osteoclastogenesis and bone

erosion and has been implicated in the process of focal bone erosion in rheumatoid arthritis (RA). However, its involvement in the immune response during chronic inflammation remains to be clarified since deficient mice show major lymph node (LN) abnormalities.
We investigated by immunohistochemistry the RANK and RANKL expression pattern of dendritic cell (DC) and T cell subsets in paired RA synovium–LN and also in normal peripheral blood mononuclear cells (PBMC) stimulated with phorbol miristate acetate/phytohemagglutin during 4, 24, and 48 hours. In RA synovium, RANKL+ cells were detected in the lining layer and the lymphocytic infiltrates, whereas RANK expression was restricted to perivascular infiltrates. In LN, RANK+ and RANKL+ cells were diffusely expressed in both the T-cell zone and germinal centers. Double staining with anti-RANK or anti-RANKL and anti-CD1a or anti-DC-LAMP antibodies showed that some immature CD1a+ DC expressed RANK and RANKL, whereas some mature DC-LAMP+ DC only expressed RANK. Double staining with the CD3, CD4 T-cell markers and the interferon gamma and IL-17 Th1 cell markers showed that some CD3+, CD4+, interferon gamma+, and IL-17+ cells produced RANKL whereas none of them express RANK. The same pattern was observed on activated PBMC. RANK+ cells were detected in unstimulated PBMC and identified as CD14+ monocytes.
This study showed the involvement of RANK/RANKL in DC–T cell interactions as found during the inflammatory process, and makes RANK and RANKL potential therapeutic targets outside the bone field.

Lipid mediators and arthritis

63 (P4.1)

The potential role of adiponectin in driving arthritis

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Background Recent data indicate that adipose tissue synthesizes and releases numerous immunocompetent molecules such as tumor necrosis factor (TNF) alpha, C3a and C1-inhibitor. As adipose tissue is also part of rheumatoid joints, we examined whether adiponectin (apM-1), which has structural homologies to complement proteins and members of the TNF family, and its receptors are present in rheumatoid arthritis (RA) and osteoarthritis (OA) synovium. In addition, owing to the ability of adiponectin to exert several immunmodulatory functions, the effect and regulation of adiponectin-dependent synthesis of proinflammatory and antiinflammatory cytokines was examined.
Methods Snap-frozen synovial sections of patients with RA, OA and articular adipose tissue were evaluated by adiponectin and adiponectin receptors type 1 and 2-specific primers, by *in situ* hybridization using adiponectin riboprobes and antibodies against adiponectin in combination with double-labeling for fibroblasts. In addition, fresh synovial fibroblasts derived from RA and OA patients undergoing knee joint endoprothetic surgery were stimulated with adiponectin in concentrations from 0.2 to 100 μg/ml for 15 hours and compared with unstimulated fibroblasts. Supernatants were examined for production of IL-1β, IL-4, IL-6, IL-10, matrix metalloproteinase (MMP)-1 and MMP-3 by commercially available ELISA.
Results In all patients, strong expression of adiponectin mRNA could be detected in synovial lining and to a lesser extent in synovial sublining. Double-labeling with anti-vimentin (fibroblasts) showed that up to 60% of RA fibroblasts expressed the adiponectin gene and adiponectin type 1 and 2 receptors. Stimulation of synovial fibroblasts with adiponectin showed a dose-dependent upregulation of IL-6 and MMP-1, both in RA and OA synovial fibroblasts. Whereas treatment without or with a small concentration of adiponectin (0.2 μg/ml) resulted only in a low production of IL-6, a concentration-dependent increase could be found with the highest amount produced with 100 μg/ml adiponectin. In contrast, synthesis of IL-1β, IL-4, and IL-10 was not altered by adiponectin stimulation. Of interest, both the TNF inhibitors adalimumab and etanercept were able to downregulate adiponectin-dependent IL-6 and MMP-1 synthesis significantly.
Conclusions The results show that adipocytokines such as adiponectin are not only present in arthritic synovium, but also that adiponectin has stimulatory effects both on the production of distinct cytokines (e.g. IL-6) and of matrix-degrading enzymes (e.g. MMP-1) in synovial fibroblasts. Therefore, the data support the hypothesis that adiponectin may alter the expression of local synovial cytokines and thus may not only represent a candidate gene of a complement/TNF-related pathway operative in RA, but also one of the targets of TNF inhibitor therapy.
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64 (P4.2)

Leptin induces production of eicosanoids and proinflammatory cytokines in human synovial fibroblasts

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Background Leptin is an adipocyte-derived hormone with an important role in regulation of feeding behavior, energy expenditure and, therefore, body weight via central nervous system mechanisms. Plasma leptin levels are reliably increased with increasing body mass index. Leptin receptors are widely distributed in peripheral tissues as well. Several different forms of leptin receptor have been identified, although the long isoform or Ob-Rb is the only isoform with clearly demonstrated signaling capability. Ob-Rb is related to the type I cytokine receptors and signals via janus-activated kinase-signal transducer and activator of transcription pathways; however, other second messenger systems may also be stimulated. A role for leptin as a modulator of the immune response and inflammatory processes has been proposed. Our group has demonstrated that leptin increases production of eicosanoids in rat macrophages. A role for leptin as an intermediary of obesity-related osteoarthritis (OA) has been proposed [1]. Leptin levels are elevated in synovial fluid of OA patients and correlate with the body mass index. Leptin and its receptor (Ob-Rb) are expressed in OA chondrocytes, and leptin treatment increases expression of transforming growth factor beta and insulin-like growth factor 1.
Aim There is a paucity of information regarding the effect of leptin on human synovial cells, and we aimed to determine whether leptin altered production of eicosanoids and proinflammatory cytokines.
Methods and results All experiments were performed in primary human synovial cells of patients with rheumatoid arthritis or OA collected at the time of surgery, and were repeated at least three times with cells from three different patients. We determined that the leptin receptor (Ob-Rb) is expressed by human synovial fibroblast-like cells using RT-PCR. Leptin receptor expression is unaffected by treatment with IL-1β (1 ng/ml). Treatment of human synovial fibroblasts with leptin (100 ng/ml) increased production of IL-6, IL-8, and prostaglandin (PG) E_2, although to a modest degree compared with IL-1β (1 ng/ml) used as a positive control. The mechanism of increased PGE_2 production involves several PG biosynthetic enzymes. We demonstrated that leptin increased phosphorylation of $cPLA_2$ by 15 min after treatment. Leptin also increased cyclooxygenase-2 protein levels. Microsomal prostaglandin E synthase 1 expression was not changed. We next evaluated the signaling pathways that may contribute to increased production of inflammatory mediators. Leptin (100 ng/ml) increased phosphorylation of p42/p44 ERK, p38, and JNK by 15–30 min after treatment, and phospho-MAPK levels were increased for 1–2 hours.
Conclusions Taken together, these data suggest that leptin may affect production of proinflammatory mediators in synovial tissues and enhance inflammation in patients with arthritis.
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65 (P4.3)

Activation of peroxisome proliferator-activated receptor γ inhibits IL-1β-induced mPGES-1 expression in human synovial fibroblasts by interfering with Egr-1

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Membrane-associated prostaglandin (PG) E synthase-1 (mPGES-1) catalyzes the conversion of PGH_2 to PGE_2, which plays a critical role in the pathogenesis of arthritis. Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated transcription factor and was shown to regulate a number of inflammatory genes in several cell types. In this study, we examined the effect of PPARγ ligands on IL-1β-induced mPGES-1 expression in human synovial fibroblasts.

The PPARγ ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and the thiazolidinedione troglitazone (TRO), but not the PPARα ligand Wy14643, dose-dependently suppressed IL-1β-induced PGE_2 production, as well as mPGES-1 protein and mRNA expression. 15d-PGJ_2 and TRO suppressed IL-1β-induced activation of the mPGES-1 promoter. Overexpression of wild-type PPARγ further enhanced, whereas overexpression of a dominant negative PPARγ alleviated, the suppressive effect of both PPARγ ligands. Furthermore, pretreatment with an antagonist of PPARγ, GW9662, relieved the suppressive effect of PPARγ ligands on mPGES-1 protein expression, suggesting that the inhibition of mPGES-1 expression is mediated by PPARγ.

Previous studies have shown that Egr-1 plays a pivotal role in transactivation of mPGES-1 gene. We demonstrated that PPARγ ligands suppressed Egr-1-mediated induction of the activities of the mPGES-1 promoter and of a synthetic reporter construct containing three tandem repeats of an Egr-1 binding site. Electrophoretic mobility shift and supershift assays for Egr-1 binding sites in the mPGES-1 promoter showed that both 15d-PGJ_2 and TRO suppressed IL-1β-induced DNA binding activity of Egr-1. This occurs without interfering with Egr-1 expression.

These data define mPGES-1 and Egr-1 as novel targets of PPARγ and provide further support for the promising application of PPARγ ligands in the treatment of arthritis.

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66 (P4.4)

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibits IL-1β-induced cyclooxygenase-2 expression in human synovial fibroblasts through a histone deacetylase-independent mechanism

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Objective 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is a natural ligand for peroxisome proliferator-activated receptor gamma (PPARγ) and has been reported to inhibit the expression of a number of inflammatory genes in several cell types. However, its effects on cyclooxygenase-2 (COX-2) expression remains controversial. In the present study, we investigated the effects of 15d-PGJ_2 on IL-1β-induced COX-2 expression in human synovial fibroblasts.

Methods COX-2 protein and mRNA expression were evaluated using western blotting and real-time PCR analysis, respectively. The COX-2 promoter activity was analyzed in transient transfection experiments. Chromatin immunoprecipitation assays were performed to evaluate the level of histone acetylation and the recruitment of HDAC1, HDAC2, HDAC3, and p300 to the COX-2 promoter.

Results 15d-PGJ_2 inhibited IL-1β-induced COX-2 protein and mRNA expression, as well as COX-2 gene promoter activation. The suppression of COX-2 protein expression was abrogated by the PPARγ antagonist, GW9662, suggesting that this effect is mediated by PPARγ. The induction of COX-2 by IL-1β is associated with hyperacetylation of histone H3 and H4 at the COX-2 promoter. Interestingly, 15d-PGJ_2 selectively blocked IL-1β-induced histone H3 acetylation. This reduction was demonstrated to not correlate with the recruitment of histone deacetylase (HDAC) to the COX-2 promoter. Also, treatment with the specific HDAC inhibitor, trichostatin A, did not relieve the suppressive effect of 15d-PGJ_2, indicating that HDACs are not involved in the inhibitory effect of 15d-PGJ_2 on COX-2 expression. Furthermore, 15d-PGJ_2 blocked IL-1β-induced recruitment of the histone acetylase (HAT) p300 to the COX-2 promoter, which may be the mechanism for decreased histone H3 acetylation and COX-2 expression. In line with this, overexpression of p300, but not of a mutant p300 lacking HAT activity, relieved the inhibitory effect of 15d-PGJ_2 on COX-2 promoter activation.

Conclusion Our data suggest that 15d-PGJ_2 can inhibit IL-1β-induced COX-2 expression in a PPARγ-dependent, HDAC-independent mechanism, probably by interfering with the HAT p300.

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67 (P4.5)

A critical role for leukotriene B_4 in K/BxN serum transfer arthritis pathogenesis

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The leukotrienes (LTs) are potent inflammatory mediators whose functions include modulation of vascular permeability, induction of adhesion molecule expression, potent leukocyte chemoattraction, stimulation of smooth muscle contraction, induction of synovial fibroblast proliferation and triggering of cytokine secretion. Knowing that these bioactivities are active in the context of inflammatory arthritis, we hypothesized that the LTs may play a critical role in the pathogenesis of murine K/BxN serum transfer arthritis. We used a genetic approach to demonstrate the functional requirement for specific LT species in the induction of K/BxN serum transfer arthritis. We find that mice lacking 5-lipoxygenase, a proximal enzyme in LT biosynthesis, are resistant to the development of arthritis. We further demonstrate that LTA_4 hydrolase-deficient mice, specifically lacking LTB_4, are also profoundly resistant to arthritis induction. In contrast, mice lacking LTC_4 synthase (and thus lacking all cysteinyl LTs), are fully competent to develop arthritis. The profound requirement for LTB_4 highlights the importance of this lipid mediator of inflammation in the orchestrated, yet poorly understood, process of arthritis. Furthermore, these observations provide an *in vivo* rationale for pursuit of therapies directed at LTs and their receptors, an inflammatory pathway not currently utilized in treatment of patients with inflammatory arthritis.

68 (P4.6)

Clusterin, a potential pathophysiological actor in rheumatoid arthritis

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Aim To ameliorate the understanding of the mechanisms involved in rheumatoid arthritis (RA) and to identify new therapeutic targets and develop novel diagnosis tools. The microarray technology allowed us to identify a set of gene specifically expressed in the synovial tissue of RA patients in comparison with osteoarthritis (OA). Among them, we

focused on clusterin since it has multiple functions related to the pathophysiological processes suspected to be involved in RA.
Results Using real-time quantitative PCR on a larger set of samples than on the microarray study, we demonstrated the highly significant ($P < 0.0001$) underexpression of clusterin in RA versus OA and versus synovial tissues of healthy individuals. The fold differential expression between OA and RA was 9.8 and between healthy tissue and RA was close to 5.2. Northern blot analysis further confirmed this differential expression and did not demonstrate an alternative splicing form of mRNA between RA and OA. Immunohistochemistry analyses of synovial tissues demonstrated that clusterin protein was mainly localised in synovial cells. The differential expression between OA and RA persisted in third-passage synovial cells. Western blot extended this differential expression at the protein level and pointed towards specific impairment of protein isoforms in RA tissues compared with OA tissues. The differential expression concerned both the intracellular and nuclear isoforms of the protein as evidenced by real-time quantitative PCR and western blot analysis.
Conclusions Due to the numerous functions associated with clusterin, the strong underexpression of clusterin mRNA in RA synovial tissue, which is associated with the specific impairment of the intracellular forms of the protein, could have tremendous effects on several pathophysiological processes taking place in RA.

T-cell regulation

69 (P5.1)

Cytokine networks in human severe combined immunodeficiency: a model for regulating T-cell homeostasis

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Objective Human T-lymphocyte homeostasis is a complex process wherein the number of T lymphocytes is held relatively constant in normal individuals. How the body senses the numbers of circulating T-cells and regulates their expansion and survival is unclear. We sought to better understand this process by profiling serum cytokines in severe combined immunodeficiency (SCID) before and after T-cell reconstitution with bone marrow transplantation.
Methods We used a novel protein microarray technology to simultaneously measure the levels of 78 different soluble proteins in the serum of 10 normal subjects and 31 SCID subjects before and at various times after transplantation. We used a linear mixed analysis of variance model and novel SAS graphics tools to identify factors that were altered in SCID and changed in response to T-cell engraftment.
Results We identified that IL-15 > MCP-1 > HCC4 > IL-7 were overexpressed in SCID patients before transplantation at times when there were few circulating T cells ($P < 0.001$ compared with controls). Both IL-7 and IL-15 normalized after successful transplantation with normal levels of circulating T cells, but not after failed transplants. HCC4 and MCP-1 levels remained high despite transplantation. Levels of nine growth factors were decreased and levels of six proinflammatory cytokines were increased after successful transplantation for SCID.
Conclusion Our studies in a human model of aberrant T-lymphocyte homeostasis wherein IL-15 and, to a lesser extent, IL-7 levels inversely correlate with circulating T-lymphocyte levels provide evidence that IL-15 and IL-7 may regulate human T-lymphocyte homeostasis. In addition, the downmodulation of numerous growth factors and upregulation of proinflammatory cytokines after successful T-cell reconstitution identify factors other than IL-15 and IL-7 that may be involved in the sensing and regulation of T-lymphocyte homeostasis in man.

70 (P5.2)

Different molecules at the surface of stimulated T cells induce IL-1α, tumor necrosis factor and IL-1 receptor antagonist in human monocytes

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An imbalance in cytokine homeostasis is thought to play an important part in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis and multiple sclerosis. We demonstrated that T cells might exert a pathological effect through direct cellular contact with monocyte-macrophages, inducing a massive upregulation of IL-1 and tumor necrosis factor (TNF). More recently, we showed that this mechanism, likely to be of relevance to chronic inflammation, is specifically inhibited by high-density lipoproteins (HDL). Like many other stimuli besides proinflammatory cytokines, the contact-mediated activation of monocytes induces the production of cytokine inhibitors such as IL-1 receptor antagonist (IL-1Ra). We observed that HDL inhibited the production of IL-1β and TNF but not that of IL-1Ra induced in monocytes after activation by membranes isolated from stimulated T cells to mimic cellular contact. This was also observed in peripheral blood mononuclear cells stimulated by either phytohemagglutinin or tetanus toxoid, in which the presence of isolated HDL inhibited the production of IL-1β and TNF while that of IL-1Ra remained unchanged. This effect was confirmed by activating isolated monocytes with CHAPS-solubilized membranes from stimulated T cells, suggesting that HDL interacted with a specific factor expressed at the surface of T cells and not unspecifically with isolated membranes. Similarly, IL-1Ra mRNA expression was not inhibited, contrary to IL-1β and TNF mRNA. This demonstrates that different molecules at the surface of stimulated HUT-78 cells are involved in the induction of IL-1β, TNF and IL-1Ra in monocytes, IL-1β and TNF being activated by one or more HDL-specific ligands. Separation of CHAPS-solubilized membrane molecules by liquid isoelectric focusing revealed two activity peaks: one activating IL-1β, TNF and IL-1Ra production; the other inducing the production of IL-1Ra in the absence of IL-1β and TNF. Thus different factors are expressed at the surface of stimulated T cells that are differently affected by HDL and differentially trigger the production of proinflammatory and antiinflammatory factors.

71 (P5.3)

Regulation of T-cell differentiation by IL-4Rα-chain single nucleotide polymorphisms

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Chronic inflammation in rheumatoid arthritis (RA) is mediated by repeatedly activated proinflammatory Th1 cells. In contrast, Th2 cells that might downmodulate the chronic autoimmune response are rarely found in RA. It has been previously documented that RA T cells are severely impaired in their ability to differentiate into Th2 effectors while exerting enhanced Th1 differentiation. The mechanisms underlying this functional abnormality, however, have not been delineated. As IL-4 is a most critical determinant in regulating immune responses by promoting Th2 cell development and inhibiting Th1 cell differentiation, we analyzed the role of single nucleotide polymorphisms (SNP) in the IL-4 receptor α-chain, which is critical for binding of IL-4 and for IL-4 signal transduction, in the differentiation of human T cells. Naive and memory CD4 T cells were isolated from the peripheral blood of 348 healthy individuals and genotyped by allele-specific PCR for the two IL-4R α-chain SNPs that are located in functionally important regions of the IL-4R α-chain – the I50V SNP50 and the Q551R SNP551 in the IL-4-binding and STAT6-binding domains, respectively. To analyze the functional role of IL-4R α-chain SNPs for T-cell differentiation, CD4-positive T cells were purified from the peripheral blood from the individuals who were homozygous for either allele at SNP50 and SNP551, and primed for 5 days with mAbs to CD28 and/or CD3 in the presence or absence

of exogenous IL-4. The phenotype of the resulting differentiated effector cells was then analyzed by flow cytometric analysis of cytoplasmic cytokines. The SNP551 alleles did not significantly affect T-cell differentiation. In marked contrast, the inhibitory effect of IL-4 on Th1 cell differentiation was significantly diminished in CD4 T cells that were homozygous for the mutated allele at SNP50 (50V) as compared with those with the wild-type allele (I50). Likewise, the augmenting effect of IL-4 on Th2 cell differentiation was markedly enhanced on T cells that were homozygous for the wild-type allele as compared with T cells expressing the mutant allele. The data indicate that the mutated allele of the IL-4R α-chain SNP50 is associated with a decreased T-cell response to IL-4. Thus, SNP50 of the IL-4R α-chain might regulate T-cell differentiation by altering T-cell responses to IL-4 and contribute to the development of unbalanced Th subset activation, as characteristic for autoimmune diseases, such as RA.

72 (P5.4)

GATA-3 in human T helper cell type 2 development

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Autoimmune inflammation, such as in rheumatoid arthritis, is characterized by activated Th1 cells without sufficient Th2 differentiation that might downmodulate the chronic immune response. Delineation of the mechanisms that control T-cell differentiation is therefore of major importance for the understanding of the pathogenesis of autoimmune diseases. The transcription factor GATA-3 has been implicated in regulating Th2 cell differentiation in murine T cells *in vitro*, but its role *in vivo* and, in particular, in human T-cell differentiation is currently unknown. To dissect the role of GATA-3 in human T-cell differentiation and T-cell-mediated effector functions, we used the unique opportunity to analyze T-cell functions in human individuals lacking one functional GATA-3 allele. The patients had no history of severe or opportunistic infections, normal peripheral T-cell counts and normal frequencies and absolute numbers of CD4 helper and CD8 cytotoxic T cells. CD4 T cells from GATA-$3^{+/-}$ individuals expressed significantly reduced levels of GATA-3, associated with markedly decreased Th2 frequencies *in vivo*, as determined by analyzing cytokine secretion profiles of freshly isolated CD4 T cells, and *in vitro*, as determined by employing an *in vitro* cell culture system that allows the differentiation of T-cell effectors after short-term priming. Moreover, Th2 cell-mediated effector functions, as assessed by serum levels of Th2-dependent immunoglobulins (IgG_4, IgE), were dramatically decreased, whereas the Th1-dependent IgG_1 was elevated compared with GATA-$3^{+/+}$ controls. Concordant with these data, silencing of GATA-3 in GATA-$3^{+/+}$ CD4 T cells with small interfering RNA significantly reduced Th2 cell differentiation. Moreover, GATA-3 mRNA levels increased under Th2-inducing conditions and decreased under Th1-inducing conditions *in vitro*. Taken together, the data strongly suggest that GATA-3 is an important transcription factor in regulating human Th2 cell differentiation *in vivo*. GATA-3 might therefore constitute a promising target for immunomodulatory treatment strategies in diseases that are characterized by biased activation of Th cell subsets, such as autoimmune diseases or allergies.

73 (P3.2)

Differential induction of IL-1β and tumor necrosis factor by CD40 ligand or cellular contact with stimulated T cells depends on the degree of maturity of human monocytes

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Cellular contact with stimulated T cells potently induces cytokine production in monocytes, a mechanism likely to be of relevance to chronic inflammation. Although the identity of the surface molecules involved in this process remains elusive, CD40 and its ligand CD40L are thought to be implicated, considering that they are expressed at the inflammatory site. To assess the involvement of CD40L we compared the activation of three different types of human monocytic cells: freshly isolated monocytes, monocytes primed with interferon gamma (IFNγ-macrophages), and THP-1 cells. These cells were activated by either membranes isolated from stimulated T cells (HUT-78 or T lymphocytes) to mimic cellular contact, by soluble extracts from isolated membranes, or by CD40L trimer (CD40LT). The production of tumor necrosis factor (TNF) and IL-1β was induced by membranes of stimulated T cells in the three types of target cells, whereas CD40LT induced TNF production in IFNγ-macrophages only. Similar results were obtained with soluble extracts of T-cell membranes, demonstrating that the difference between membranes and CD40LT was not due to the particulate form of membranes. CD40LT induced neither transcript nor protein of cytokines in monocytes, whereas in IFNγ-macrophages IL-1β and TNF mRNA were observed, only TNF being detected in cell supernatants. Finally, anti-CD40L antibodies failed to inhibit TNF and IL-1β production induced in IFNγ-macrophages by solubilized membranes, while TNF production induced by CD40LT was inhibited. These results demonstrate that CD40L is not required in monocyte activation by direct cellular contact with stimulated T cells, although soluble CD40LT induces the production of TNF in IFNγ-macrophages.

Other arthritic diseases

74 (P6.1)

Clinical classification of fibromyalgia according to disease progression

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Introduction The number of fibromyalgia patients has recently been increasing in Japan. We currently studied numerous patients with early to severe neuropathic pain on not only 'specific sites' according to American College of Rheumatology ACR-90 criteria, but also widespread severe pain such as hyperpathia or allodynia.

Symptoms in these patients are sometimes accompanied with extramuscular symptoms such as irritable colon, interstitial cystitis, severe dry eye and mouth, and various psychogenic symptoms. Twenty-seven patients with severe and systemic complications were misdiagnosed with other psychogenic disorders. We classified patients with fibromyalgia in five categories according to disease progression.

Patients and method A total 153 patients with fibromyalgia who visited our rheumatology or psychiatry clinics in the past 2 years were assessed; 132 (86.5%) female patients and 21 (13.5%) male patients. The mean age was 52.3 ± 15.0 years for females, 50.4 ± 18.6 years for males. These patients were classified into five stages according to clinical symptoms (Table 1).

Results and discussion As shown in Table 1, 64 patients fulfilled the ACR-90 criteria. Patients classified in stages II–IV had painful symptoms that spread through the whole body and were extremely severe. Eight of 153 patients had extramuscular systemic symptoms. The results of the SPECT study revealed that 80% of the 40 patients clearly identified a low level in regional blood flow. Twenty-seven cases of the patients were misdiagnosed with psychogenic or neurological

Table 1

Classification of fibromyalgia according to disease progression frequency in 153 cases

Stage I	Existence of pressure pain on specific sites according to ACR-90 criteria	64 (41.8%)
Stage II	Expanding of pain from trunks to extremities and also changing nature of pain from pressure pain to spontaneous pain	46 (30.0%)
Stage III	Persistent pain is generalized and induction of burning pain by mild change of temperature, stimulation on nail and hair, and so on	16 (10.5%)
Stage IV	Sleep disturbance, restless, interfere keeping same postulation by hyperpathia, severe decreasing quality of life, allodynia	16 (10.5%)
Stage V	Severe pain is generalized and complicated tremendous extramusculoskeletal symptoms such as irritable colon, bladder, dry eye, dry mouth and mucocutaneous symptoms	11 (7.2%)

disorders for a long time. Based on such clinical diversity, it is essential to classify fibromyalgia according to disease progression.

75 (P6.2)
Raynaud's phenomenon secondary to rheumatoid arthritis may be predictive of more erosive disease

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Purpose Raynaud's phenomenon (RP) occurs less frequently in rheumatoid arthritis (RA) than with other connective tissue diseases, such as scleroderma and systemic lupus erythematosus. We studied the relationship between RP and other disease characteristics common to RA to determine whether RP can be predictive of more severe disease. Secondary analyses were preformed to assess correlations between other antibodies/manifestations that occur in RA, and the onset of RP versus RA disease duration.

Methods Using a standardized assessment, data were collected on a cross-sectional cohort of RA subjects ($n = 329$; mean age 60.3 ± 0.7 years; 77% female; 76% erosions, 75% positive rheumatoid factor [RF]) who met the American College of Rheumatology criteria for RA and had been seen at a London, Ontario rheumatology clinical practice during the 6-month study period. Study participants were prevalent (follow-up) cases and new referrals. A subsequent chart review was performed to verify clinic data and also to collect data on all variables that were not available at the time of the clinic visit. RP was defined as pallor of the fingers along with rubor, cyanosis, or both.

Results The mean disease duration was 12 ± 0.6 years. Seventy patients (22%) had RP. RP status was not related to gender, age, or disease duration. The mean age at onset of RP was 50.7 ± 2 years and the mean RP duration was 9.2 ± 1.5 years. Patients presented with RP a mean of 3.8 ± 1.4 years after the diagnosis of RA (95% confidence interval, 0.9–6.6 years; minimum, maximum = 31 years before RA diagnosis, 32 years after). RP status was not associated with the presence of nodules and erosions. Patients with sclerodactyly [all was distal to proximal interphalangeal joints] were more likely than those without to have RP (34% versus 17%, $P < 0.001$). Subjects with sclerodactyly (26%) were also more likely than those without to have erosions (86% versus 72%, $P < 0.02$). Patients who developed RP after their RA diagnosis were more likely to have erosions than those who developed RP before RA ($P < 0.005$). As expected, positive RF was associated with longer disease duration ($P < 0.04$). Higher RF values were associated with longer disease duration ($P < 0.005$) and increased RP duration ($P < 0.01$).

Conclusion RP was present in 22% of the RA patients seen in a rheumatology clinical practice in London, Ontario during the 6-month study period. RP appears to develop relatively soon (approximately 1–7 years) after RA diagnosis in the majority of cases. Idiopathic RP may be different from RP secondary to RA, the latter of which may be associated with more erosive RA. Sclerodactyly is associated with erosive arthritis and RP in RA. Higher RF values were indicative of increased RA and RP duration.

76 (P6.3)
The role of TLR4 and neutrophil expression in infection-triggered arthritis

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Background The initial response to *Chlamydia*-induced arthritis probably involves innate immunity but the nature of this interaction has not been defined. In the present study, we examined the role of neutrophils in experimental arthritis in mice with targeted elimination of the small GTPAases Rac1 and Rac2, and the role of toll-like receptors in this model.

Methods Arthritis was induced in either wild-type or Rac-deficient mice by intra-articular inoculation of synoviocyte-packaged *Chlamydia trachomatis*. The scoring of arthritis was assessed by joint swelling and quantitative histopathological scoring. Immunohistochemistry was used to determine the infiltration of neutrophils into the joint. The persistence of *Chlamydia* in joints of mice after injection was determined by immunoassay. The expression of TLR2 and TLR4 in neutrophils was detected by semiquantitative PCR. Mice genetically deficient in TLR4 were also assessed.

Results In the acute phase, wild-type mice developed more severe arthritis than Rac-deficient mice. At this stage there was abundant infiltration of neutrophils into the joint. In the chronic phase, the Rac-deficient mice developed more severe arthritis and these mice demonstrated defective clearance of the pathogen from the joint. *In vitro* stimulation of neutrophils with *Chlamydia* upregulated expression of TLR4 but not TLR3 in wild-type mice. However, neutrophils from Rac-deficient mice did not show this upregulation of TLR4. Sustained TLR4 expression in neutrophils was found to be dependent on expression of Rac. We examined mice genetically deficient in TLR4 expression and demonstrated that such mice developed more severe arthritis than controls. Thus Rac expression plays a profound role in infection-triggered arthritis and demonstrates a bimodal influence on the disease process, exacerabating acute joint inflammation but controlling chronic arthritis. Rac-deficiency was associated with diminished TLR4 expression, impaired host clearance of the pathogen and more severe chronic arthritis.

Conclusions In infection-triggered arthritis, innate immunity plays a critical role. Effective host clearance of an arthritogenic pathogen depends on intact Rac expression by neutrophils and by appropriation of TLR4 by these cells. A defect in this pathway of host defence profoundly influences the outcome of the infection. This study also highlights the changing microenvironment of the joint over time with implications for therapeutic approaches to arthritis.

77 (P6.4)

Inhibition of complement activation: a novel mechanism for the protective effects of heparin in antiphospholipid antibody-induced pregnancy loss

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The antiphospholipid syndrome (APS), defined by thrombosis and recurrent pregnancy loss in the presence of antiphospholipid (aPL) antibodies, is generally treated with anticoagulation. Because complement activation is essential and causative in aPL antibody-induced fetal injury, we hypothesized that heparin protects mice from fetal loss in APS by preventing complement activation on trophoblasts and that anticoagulation, *per se*, is not sufficient to prevent miscarriage. We used a murine model of APS in which pregnant mice are injected with human IgG-containing aPL antibodies (aPL-IgG) or IgG from healthy individuals (NH-IgG). Passive transfer of aPL-IgG caused a 43.3 ± 3.5% frequency of fetal resorption ($P < 0.001$ versus NH-IgG) and treatment with either unfractionated heparin (UFH) (10 U or 20 U subcutaneously twice daily) or low molecular weight heparin (LMWH) (enoxaparin) reduced the frequency of fetal resorption to that of mice treated with NH-IgG: UFH10 + aPL, 9.6 ± 3.2; UFH20 + aPL 10.4 ± 2.1; LMWH + aPL, 11.8 ± 2.8; NH-IgG, 10.5 ± 2.5% ($P < 0.01$ versus aPL). Even in the absence of detectable anticoagulation, as in mice treated with 10 U UFH, heparins prevented aPL-induced pregnancy loss and inhibited systemic complement activation evidenced by generation of C3adesArg (aPL, 1212 ± 101* ng/ml; UFH10U + aPL, 178 ± 25; LMWH + aPL, 180 ± 15; NH-IgG, 215 ± 381, * $P < 0.05$ versus NH-IgG). Heparin limited C3 deposition in deciduas of mice treated with aPL. Neither fondaparinux nor hirudin inhibited the generation of complement split products or prevented pregnancy loss, despite anticoagulation levels comparable with heparin, indicating that anticoagulation is insufficient therapy for APS-associated miscarriage.

We assessed the effects of anticoagulants on complement activation *in vitro* using BeWo cells, trophoblast-like cells with externalized phosphatidylserine recognized by aPL. APL-opsonized BeWo cells cultured with mouse serum activated complement, evidenced by increased surface-bound C3 fragments (detected as C3 staining by FACS) and generation of soluble C3adesArg in supernatants. In the presence of UFH or LMWH, however, complement activation was completely inhibited (C3adesArg [ng/ml]: aPL, 680 ± 30; UFH + aPL, 120 ± 20*; LMWH + aPL, 105 ± 18*; % C3-positive BeWo cells by FACS: aPL, 31 ± 11; aPL + UFH, 9 ± 5*; LMWH + aPL, 5 ± 3; * $P < 0.01$ versus aPL). In contrast, neither fondaparinux nor hirudin inhibited complement activation *in vitro*.

In conclusion, heparins inhibit complement activation *in vitro* and *in vivo*. Heparins may prevent obstetrical complications in APS by blocking activation of complement induced by aPL targeted to deciduas – not by preventing placental thrombosis. These findings explain how subanticoagulant doses of heparin can limit antibody-mediated tissue injury.

Session B

Osteoarthritis: pathogenesis/growth factors

78 (P7.1)

Advanced glycation endproducts in the development of osteoarthritis

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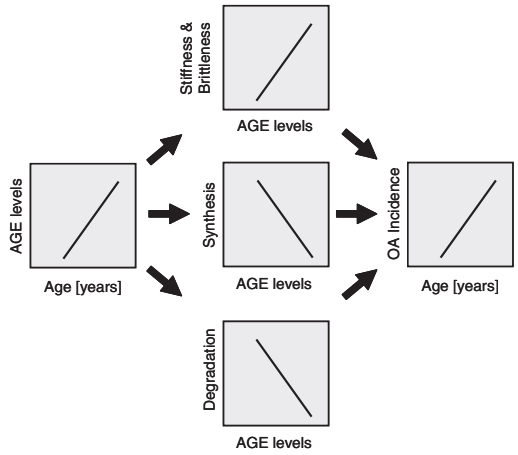
Background Osteoarthritis (OA) is one of the most prevalent and disabling chronic conditions affecting the elderly. The most prominent feature of OA is the progressive destruction of articular cartilage resulting in impaired joint motion, severe pain and, ultimately, disability. Age is identified as the main risk factor for the development of OA, but the mechanism by which aging is involved still remains largely unclear. Age-related changes in the articular cartilage could play an important role in the susceptibility of cartilage to OA. One of the major age-related changes in articular cartilage is the accumulation of advanced glycation endproducts (AGEs), resulting from the spontaneous reaction of reducing sugars with proteins. The present studies were designed to investigate whether AGE accumulation in cartilage may predispose to the development of OA.

Figure 1



Hypothesis of how advanced glycation endproduct (AGE)-related accumulation of AGEs could predispose to the development of osteoarthritis (OA).

Methods The role of AGEs in the development of OA was studied by a combination of *in vitro*, *ex vivo* and *in vivo* experiments. The type and quantity of AGEs in human articular cartilage were determined using HPLC and GC-MS methods. Effects of AGE accumulation on cartilage extracellular matrix turnover were assessed in human articular cartilage and bovine alginate cultures using radiolabel incorporation, colorimetric, enzyme activity and HPLC analyses. The *in vivo* role of AGEs in OA predisposition was studied in the canine ACLT model for OA.

Results High levels of all well-characterized AGEs (pentosidine, carboxymethyllysine and carboxyethyllysine) accumulate with age in cartilage collagen. Furthermore, an age-related increase of general measures of AGEs (fluorescence at 370/440 nm, browning, and amino acid modification) was also observed [1]. Accumulation of AGEs was correlated with increased stiffness and brittleness of the cartilage, rendering it more prone to mechanical damage. In addition to affecting the mechanical properties of tissues, articular cartilage chondrocytes show decreased proteoglycan and collagen synthesis at increased AGE levels. Degradation of AGE-modified collagen by matrix metalloproteinases is impaired compared with unmodified collagen. In a canine study of experimentally induced OA by anterior cruciate ligament transection, animals with elevated AGE levels suffered from more severe OA than those with normal AGE levels [2]. Moreover, in a cross-sectional study using human articular cartilage samples obtained at autopsy, the presence of cartilage degeneration was associated with higher AGE levels in the joint cartilage.

Conclusion AGE accumulation in cartilage leads to decreased mechanical properties (increased stiffness and brittleness) and impaired extracellular matrix turnover (decreased synthesis and degradation). Together these data support the hypothesis presented in Fig. 1 that the age-related accumulation of AGEs changes the properties of articular cartilage and thereby renders the tissue more prone to the development of OA.

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79 (P7.2)

New emerging roles of transcription factor Pitx1 and Reg growth factors in osteoarthritis pathogenesis

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Background The key features of osteoarthritis (OA) are the focal destruction of the articular cartilage and the abnormal growth of the subchondral bone producing outgrowths. Since in humans OA develops and changes very slowly, it is difficult to follow that disease over any length of time. Besides that, the heterogeneity of the disease results in controversy as regards its aetiology and progression. Thus, study of early events of the degenerative process cannot be made in humans and recourse must be made to animal models. We have recently inactivated the transcription factor Pitx1, which is highly expressed in articular and growth plate chondrocytes during mouse development. Pitx1-null mice displayed poorly developed joints, which are markedly apoptotic. Interestingly, Pitx1$^{+/-}$ mice that are phenotypically normal at birth exhibit with aging clinical features of OA such as progressive joint stiffness associated with an abnormal fibrillation and calcification of their articular cartilage. Histological analysis of mineralized adult femurs and tibias revealed also an increased thickening of subchondral, trabecular and cortical bone. At the molecular level, expression analysis of Pitx1-null and Pitx1$^{+/-}$ mice allowed one to identify and characterize a novel molecular cascade involved in OA pathogenesis.
Objective To assess whether a loss-of-function of Pitx1 transcription factor in human articular chondrocytes and the subsequent activation of Reg growth factors trigger OA pathogenesis.
Methods RNA isolated from articular cartilage of Pitx1-null mice and from patients with OA and age-matched and gender-matched subjects were prepared to perform a molecular expression analysis. In parallel, cartilage explants were analyzed by immunohistochemistry method to detect the presence of Reg I proteins, and transient transfection assays were performed to assess the contribution of Reg growth factors in the activation of NF-κB induced by proinflammatory cytokines in OA.
Results Expression analysis showed the expression of Pitx1 only in matched controls. Moreover, the lack of Pitx1 in OA articular chondrocytes leads to a marked up regulation of Reg I growth factor and its receptor, which has been confirmed at the protein level by immunohistochemistry assays with human Reg I antibodies on OA articular cartilage. We have also demonstrated *in vitro* that the gain-of-function of Reg receptor enhances by several fold the activation of NF-κB induced by tumor necrosis factor alpha or IL-1β, suggesting that Reg signaling activity is a key mediator of proinflammatory cytokine action in OA pathogenesis. This was further supported by the fact that gain-of-function of Pitx1 abrogates Reg I, Reg II and Reg receptor expression completely, which may explain why proinflammatory cytokines cannot activate NF-κB in cells devoid of Reg receptor.
Conclusion Taken together these results revealed the role of new emerging transcription and growth factors involved in OA pathogenesis, which could lead to a more rational approach for the development of better therapeutic compounds to prevent and cure OA.

80 (P7.3)

Differential gene expression and regulation of the bone morphogenic protein antagonists follistatin and gremlin in normal and osteoarthritic human chondrocytes and synovial fibroblasts

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Objective To compare gene expression and regulation of the bone morphogenic protein (BMP) antagonists follistatin, gremlin, chordin and noggin in human normal and osteoarthritis (OA) chondrocytes and synovial fibroblasts.
Methods Follistatin protein production was measured using a specific ELISA, and localization of follistatin and gremlin in cartilage was determined by immunohistochemistry. Basal and induced gene expression was determined using real-time PCR. Gene expression was monitored following treatment with inflammatory, antiinflammatory, growth and developmental factors.
Results All BMP antagonists, except noggin, were expressed in chondrocytes and synovial fibroblasts. Follistatin and gremlin were significantly upregulated in OA chondrocytes, but not in OA synovial fibroblasts. Chordin was weakly expressed in normal and OA cells. Production of follistatin protein paralleled the gene expression pattern. Follistatin was expressed preferentially by the chondrocytes at the superficial zone of cartilage. Gremlin was not detected in normal cartilage; in OA it was found at the superficial zone, not at the very superficial layers of cartilage but rather at the upper intermediary layers. Tumor necrosis factor alpha and interferon gamma stimulated follistatin expression, but downregulated gremlin. IL-1β had no effect on follistatin, but reduced gremlin. Conversely, BMP-2 and BMP-4 significantly stimulated gremlin, but downregulated follistatin. IL-13, dexamethasone, transforming growth factor beta1, basic fibroblast growth factor, platelet-derived growth factor BB and epidermal growth factor downregulated the expression of both antagonists.
Conclusion We show, for the first time, the involvement of the BMP antagonists follistatin and gremlin in OA pathophysiology. Data suggest that follistatin and gremlin expression is timed with specific stages in the progression of OA. The balance of BMP antagonist levels during the OA process may play a critical role in influencing the progression of OA, making these antagonists interesting new targets for the treatment of this disease.

81 (P7.4)

Tumor necrosis factor alpha downregulates bone morphogenetic protein expression and matrix macromolecule synthesis in articular cartilage

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Objective Cartilage breakdown in arthritis is thought to result from disequilibrium of catabolic and anabolic mechanisms. Tumor necrosis factor (TNF) alpha is a potent stimulator of catabolic pathways, whereas its influence on anabolic pathways is currently unknown. Herein, we studied the effect of systemic overexpression of TNF on bone morphogenic protein (BMP) expression in articular cartilage and cartilage matrix synthesis.
Methods Analyses were performed in human tumor necrosis factor transgenic (hTNFtg) mice, which suffer from chronic destructive arthritis, and wild-type mice. Expression of cartilage-derived morphogenetic protein (CDMP)-1, CDMP-2, and BMP-6 and BMP-7 in articular cartilage was assessed by immunohistochemistry. Cartilage samples from the knee joints were assessed for DNA content and [^{35}S]sulfate incorporation assays to assess chondrocyte number and matrix synthesis, respectively.

Results Expression of all four BMP family members was significantly decreased in articular cartilage of hTNFtg mice. The numbers of stained cells were reduced by about 60% for CDMP-1, CDMP-2 and BMP-6 ($P < 0.004$) and by 44% for BMP-7 ($P < 0.02$). There was no difference in DNA content in the investigated cartilage samples, whereas isotope incorporation into newly synthesized matrix macromolecules was significantly decreased by an average of 61% in cartilage derived from hTNFtg mice compared with wild-type controls ($P < 0.001$).
Conclusion Chronic overexpression of TNF leads to decreased expression of BMPs and reduced matrix macromolecule synthesis in the articular cartilage. These data suggest that TNF, aside from functioning as a catabolic mediator, potently inhibits anabolic mechanisms, which aim to restore the integrity of articular cartilage.

82 (P7.5)

Attachment of synovial fibroblasts to type 1 laminin boosts the transforming growth factor beta-induced expression of stromelysin-1 (MMP-3)

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Background and objective In previous studies, the effects of attachment of synovial fibroblasts (SF) to different matrix compounds such as type I collagen or fibronectin on IL-16 expression were investigated. The focus of this study is the analysis of gene expression in SF upon stimulation with transforming growth factor beta (TGF-β) after attachment to LN1-laminin (EHS laminin).
Methods Expression of IL-1α, IL-1β, IL-6, IL-8, IL-16, IL-18 as well as matrix metalloproteinase (MMP)-1 and MMP-3 were investigated in SF from rheumatoid arthritis patients ($n = 6$) or osteoarthritis patients ($n = 5$) in primary or early passage cultures. Cells were derived from biopsies and expanded in DMEM + 10% FCS medium. Fibroblasts were seeded onto LN1-laminin-coated vessels (BD BioCoat®) for 24–72 hours, and cells attached to cell culture vessels served as controls in all experiments. After these incubations, transcript amounts of individual genes were enumerated by quantitative RT-PCR. A recombinant cytokine standard and GAPDH RT-PCR served as controls in each sample. Expression of the α1, β1 and γ1 chains of LN-1 laminin by SF was investigated by RT-PCR and immunocytochemistry.
Results Growth of SF on LN1-laminin coated surfaces without additional stimuli induced a significant IL-8 response (3.1-fold, ± 1.24; $P \leq 0.001$) and lower responses for IL-1β, IL-16, IL-1α, IL-18 and IL-6. MMP-1 was upregulated 2.3-fold (± 0.79, $P \leq 0.001$), MMP-3 only 1.5-fold. Upon incubation of SF on LN10-laminin, the cytokine and MMP expression were not changed. Addition of TGF-β (10 ng/ml, 24 hours) to SF attached to tissue culture vessels showed a different induction profile. Here IL-6 showed the most prominent induction (4.1-fold, ± 3.6; $P \leq 0.21$), IL-1β, IL-α, IL-8, IL-16 and MMP-1 were induced to a lesser degree, and IL-18 mRNA was lower whereas MMP-3 was induced (3.15-fold, ± 0.7; $P \leq 0.04$), when compared with controls. Next, the combination of activation by TGF-β and laminin signaling were investigated. For cytokine expressions, no additive effects of combining these signals were seen and MMP-1 expression was induced only to some extent (3-fold, ± 1.76). In contrast, MMP-3 was induced more than 10-fold. In SF mRNA encoding α1, β1 and γ1 laminin, which encode the proteins for LN1-laminin, were detected by RT-PCR – whereas α5 laminin mRNA, encoding the α-chain of LN10-laminin, remained undetectable by RT-PCR. Using an anti-EHS serum, LN1-laminin was detected on SF by immunocytochemistry. However, using monoclonal antibodies to laminin α1 or γ1 proteins, staining signals were very weak.
Conclusions Attachment to LN1-laminin in the presence of TGF-β may induce elevated MMP-3 expression in SF. An autocrine stimulation of MMP-3 expression by SF via TGF-β and LN1-laminin seems rather unlikely, as LN1-laminin is not expressed in high amounts in the adult synovial membrane. Still, activation of SF by LN1-laminin may serve as a model for activation of fibroblasts by extracellular matrix compounds in the presence of growth factors or cytokines, and both pathways contribute to the aggressive invasive growth of SF in the course of rheumatoid arthritis.

83 (P7.6)

Expression and regulation of microsomal prostaglandin E synthase-1 in human osteoarthritic cartilage and chondrocytes

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Objective Elevated production of prostaglandin (PG) E_2 plays an important role in the pathogenesis of arthritis. Recently, an inducible microsomal prostaglandin E synthase-1 (mPGES-1) was identified. This enzyme is functionally coupled with cyclooxygenase-2 and converts the cyclooxygenase product PGH_2 to PGE_2. In the present study we analyzed the expression of mPGES-1 in human normal and osteoarthritic (OA) cartilage and determined the effect of different inflammatory agonists on the expression of mPGES-1 in OA chondrocytes.
Methods Expression of mPGES-1 mRNA and protein in cartilage was determined by quantitative real-time RT-PCR and immunohistochemistry, respectively. OA chondrocytes were treated with different inflammatory agents and mPGES-1 protein expression was evaluated by western blot. Activation of the mPGES-1 promoter was assessed in transient transfection experiments.
Results Levels of mPGES-1 mRNA and protein were markedly elevated in OA versus normal cartilage. Treatment of chondrocytes with IL-1β induced the expression of mPGES-1 protein in a dose-dependent and time-dependent manner. This appears to occur at the transcriptional level as IL-1β induced the expression of mPGES-1 mRNA and the activity of this gene promoter. Tumor necrosis factor alpha and IL-17 also upregulated the expression of mPGES-1 protein and displayed a synergistic effect with IL-1β. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 inhibited IL-1β-induced mPGES-1 protein expression, an effect that was reversed by exogenous PGE_2.
Conclusion This study shows for the first time that mPGES-1 expression is upregulated in OA versus normal cartilage and that proinflammatory cytokines increased mPGES-1 expression in chondrocytes. These data suggest that mPGES-1 may prove to be an interesting therapeutic target for controlling PGE_2 synthesis.
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Rheumatoid arthritis: bone cell regulation

84 (P8.1)

Arthritis induces lymphocytic bone marrow inflammation and endosteal bone formation

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Imaging studies have shown that bone marrow changes occur in patients with rheumatoid arthritis (RA). To address whether bone marrow is affected in the course of arthritis, human tumor necrosis factor transgenic (hTNFtg) mice, constituting an established animal model of human RA, were examined for bone marrow changes. The hind paws (tarsal area) of 22 untreated hTNFtg mice, of five hTNFtg mice treated with anti-tumor necrosis factor (infliximab) and of five wild-type mice were examined histologically, immunohistochemically and by means of mRNA *in situ* hybridization. All untreated hTNFtg mice with moderate ($n = 10$) and severe ($n = 7$) disease developed inflammatory bone marrow lesions during the course of disease, whereas no such lesions appeared in hTNFtg mice with mild disease ($n = 5$) and in wild-type mice. Bone marrow infiltrates were almost exclusively composed of lymphocytes and the overwhelming proportion (> 80%) were B

cells. The presence and extent of bone marrow infiltrates were closely linked to severity of arthritis. In addition, blockade of tumor necrosis factor effectively reduced bone marrow inflammation. Interestingly, osteoblast numbers were increased at the endosteal surface in the vicinity of these lesions. Moreover, osteoid deposition, expression of bone matrix proteins, such as osteocalcin and osteopontin, and mineralization were enhanced, suggesting that inflammatory bone marrow infiltrates induce bone formation. Indeed, B lymphocytes of these lesions expressed bone morphogenetic protein (BMP)-6 and BMP-7, which are important stimulators of new bone formation. Thus, we conclude that bone marrow actively participates in destructive arthritis by generating B-lymphocyte-rich bone marrow lesions and inducing endosteal bone formation.

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85 (P8.2)

Distinct lymphocyte subpopulations in bone marrow from rheumatoid arthritis and osteoarthritis patients: a role for IL-15?

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Introduction Recent data indicate that bone marrow plays an important role not only as a primary lymphoid organ responsible for haemopoiesis, but also as a secondary lymphoid organ with capability of antigen presentation exceeding that of lymph nodes. Although in chronic inflammatory/immune disease, like rheumatoid arthritis (RA), bone marrow participates in the initiation and/or perpetuation of the disease, there is little information about the real number of lymphocyte subpopulations in bone marrow of these patients and how they can be modulated by T-cell growth factors. IL-15 acting through IL-15 receptors (including the high-affinity IL-15R alpha chain) is a key cytokine influencing the development of natural killer cells in bone marrow, and proliferation and maintenance of the memory T-cell pool. However, there is no information about the levels of IL-15 in bone marrow.

Objective In the present study we measured the real numbers of lymphocyte subsets in bone marrow isolated from RA and osteoarthritis (OA) patients in correlation with the levels of soluble IL-15 and surface-expressed IL-15R alpha.

Methods Bone marrow samples, obtained from nine RA and nine OA patients (mean age 53.1 ± 10.6 years and 54.3 ± 13.6 years, respectively) undergoing joint replacement surgery, were diluted four times in heparinized PBS. Bone marrow plasma samples were obtained by centrifugation and levels of IL-15 were measured using specific ELISA. The real number of lymphocytes stained for $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ were counted in the presence of TruCount beads using flow cytometry. Surface-expressed IL-15R was done on cells separated by gradient centrifugation, acid wash of surface-bound IL-15 and flow cytometric analysis.

Results The real number of $CD3^+$, $CD4^+$, $CD8^+$ T cells and $CD19^+$ B cells, and statistical significance of these data are presented in Table 1. There were twice as many T ($CD3^+$) cells in RA in comparison with OA bone marrow. In contrast, only 42% of B ($CD19^+$) cells present in OA were observed in RA. Interestingly, lymphocytes isolated from RA patients expressed a significantly higher level of surface IL-15R alpha chain, indicating their activation status. In addition, there is a tendency (although not statistically significant, $P = 0.08$) for elevated levels of IL-15 in bone marrow plasma from RA in comparison with OA patients (2390 ± 1687 pg/ml and 1292 ± 457 pg/ml, respectively).

Conclusion A highly significant increase of $CD3^+$ (both $CD4^+$ and $CD8^+$) T-cell numbers in RA in comparison with OA suggest that T cells in RA are actively trafficking to bone marrow or vigorously proliferate *in situ*, or both. Since lymphocytes from RA, in contrast to OA, express IL-15 receptors, and since there is a tendency to higher levels of IL-15 in RA, it is likely that T cells actively proliferate in bone marrow in response to locally produced IL-15. Significantly lower B-cell numbers in RA than in OA suggest that these cells actively emigrate from RA bone marrow to peripheral blood and affected joints.

Table 1

Real number of lymphocytes isolated from bone marrow of rheumatoid arthritis (RA) and osteoarthritis (OA) patients

Patients	$CD3^+$	$CD4^+$	$CD8^+$	$CD19^+$
RA	6.1 ± 2.8	3.0 ± 1.2	3.0 ± 1.7	0.85 ± 0.3
OA	3.2 ± 1.6	1.6 ± 0.8	1.6 ± 0.9	2.0 ± 0.9
RA versus OA	$P < 0.008$	$P < 0.004$	$P < 0.03$	$P < 0.02$

Data presented as cell number × 10^6/ml bone marrow.

86 (P8.3)

Suppression of cbfa1 expression in C3H10T1/2 mesenchymal stem cells by anti-cbfa1 siRNA blocks osteoblastic develoment, but does not affect chondrogenesis

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Introduction Mesenchymal stem cells (MSC) may differentiate into adipocytes, osteoblasts or chondrocytes, the latter leading to hypertrophic chondrocytes responsible for endochondral ossification. The presence of the transcription factor cbfa1 is a prerequisite for osteoblast development, but is also of major importance in chondrocyte transition to the hypertrophic state. For cartilage tissue engineering, we need to prevent hypertrophic phenotype of MSC differentiated to chondrocytes. For this, we transfected mesenchymal stem cells transiently with anti-cbfa1 siRNA and investigated its effect on bone morphogenic protein (BMP)-2-induced osteogenesis in monolayer culture, or chondrogenesis in cultured micropellets.

Methods We used the murine MSC C3H10T1/2 to obtain chondrogenic differentiation in micropellets. In order to assess impact of cbfa1 inhibition, we used a plasmid encoding the osteocalcin promoter coupled to the luciferase gene. Cells were transfected using 1–30 pmoles siRNA designed to inhibit cbfa1 per 10,000 cells and oligofectamine according to standard protocols, and thereafter incubated for up to 28 days.

Results It appeared that both the cbfa1 expression (RT-PCR) and nuclear protein levels (immunohistochemistry) were diminished up to 75% at day 5. Furthermore, cells transfected with a plasmid containing the osteocalcin promoter coupled to the luciferase gene displayed an approximate 65% reduction in luciferase activity subsequent to transient exposure to anti-cbfa1 siRNA. cbfa1 suppression also markedly reduced the expression of several osteoblast-related genes (i.e. osterix; collagen-I; alkaline phosphatase; osteocalcin; secreted protein acidic, cysteine-rich or osteonectin; and osteopontin).

Functional parameters such as enzymatic activity of alkaline phosphatase and mineralization surface profiles over a period of 28 days were both delayed by a mean of 8 days. In contrast, cells differentiated to chondrocytes in micropellets and transfected with the siRNA duplex did not display altered expression of aggrecan or collagen II, as compared with nontransfected cells.

Conclusion In summary, these experiments indicate that a transient and substantial suppression of cbfa1 in C3H10T1/2 cells, grown in the presence of BMP-2, is sufficient to delay mineralization, while not reducing their ability to differentiate towards functional chondrocytes. Vectors to express anti-cbfa1 siRNA and to stably transfect stem cells are in progress.

87 (P8.4)

Abnormal collagen type I production in osteoarthritic subchondral bone is associated with a reduced capacity of osteoblasts to mineralize *in vitro*

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Background Osteoarthritis (OA) is characterized by cartilage loss, synovial inflammation, osteophytes, and abnormal subchondral bone remodeling including sclerosis. Bone sclerosis in OA is due to an abundant osteoid collagen matrix. Collagen type 1 synthesis is increased in *in vivo* OA bone tissue and there is an abnormal ratio of collagen type 1α1 chains (Coll1α1) to Coll1α2 chains in this tissue. The mechanisms responsible for this abnormal osteoid matrix remain unknown.

Objective In this study using *in vitro* subchondral osteoblasts (Ob) from normal and OA individuals, we investigated the mechanisms responsible for abnormal collagen production.

Methods We used primary human subchondral Ob from normal and OA individuals. Cells were stimulated or not with 100 ng/ml parathyroid hormone (PTH), 500 nM prostaglandin E_2 (PGE_2) or 50 nM $1,25(OH)_2D_3$. RNA was extracted with TRIzol and used to perform RT-PCR and real-time PCR of Coll1A1 and Coll1A2. Coll1 synthesis was assessed as the release of the carboxy terminal peptide fragment (CICP), which reflects *de novo* collagen synthesis. Proteins were separated by SDS-PAGE and detected using selective antibodies against Coll1α1, Coll1α2, or membrane-type 1 or membrane-type 2 matrix metalloprotease (MT1-MMP and MT2-MMP). MMP-2 and MMP-9 activities were assessed by zymography. Mineralization was evaluated by the von Kossa staining of cells after 30 days of culture in the presence or not of 10 ng/ml bone morphogenic protein-2 (BMP-2).

Results Data showed that basal Coll1A1 mRNA levels were significantly increased in OA Ob compared with normal using real-time PCR, whereas Coll1A2 levels in OA Ob were similar to normal. This translated into an α1 to α2 collagen type I ratio of 2.5 in normal Ob whereas it increased to 7.2 in OA Ob. PTH and PGE_2 both reduced Coll1A1 and Coll1A2 mRNA levels in normal Ob yet this was reduced for OA Ob. Indeed, PGE_2 reduced Coll1A1 and Coll1A1 mRNA levels about half as in normal, and the effect of PTH was virtually absent in OA Ob. Basal collagen type I synthesis, determined by the release of the C-terminal propeptide and by western blot analysis, was also higher in OA Ob than normal. MMP-2 and MMP-9 were increased in OA Ob compared with normal as determined by zymography. Western blot analysis showed an increase in MT2-MMP but not in MT1-MMP in OA Ob. Finally, the mineralization of OA Ob was significantly reduced compared with normal as determined by Von Kossa staining under both basal conditions and following BMP-2 stimulation.

Conclusion These results suggest that a cellular defect of OA Ob and an abnormal response to PTH and PGE_2 challenge could explain abnormal production and ratio of α1 to α2 chains of mature collagen type 1 in these cells. Coupled to the increase in MMP activities, this could explain the abnormal collagen remodeling observed in OA bone tissue *in vivo*.

88 (P8.5)

Zoledronic acid protects from local and systemic bone loss in tumor necrosis factor-mediated arthritis

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

Human tumor necrosis factor transgenic (hTNFtg) mice, which develop severe destructive arthritis as well as osteoporosis, were treated with PBS, single or repeated doses of ZA, calcitonin or anti-tumor necrosis factor at the onset of arthritis.

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

ZA appears as an effective tool to protect bone from arthritic damage. In addition to antiinflammatory drug therapy, modern bisphosphonates are promising candidates to maintain joint integrity and to reverse systemic bone loss in arthritis.

89 (P8.6)

Rheumatoid arthritis and risk factors for low bone mineral density

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Objective To study the influence of gender, menopausal status, smoking, previous nonvertebral fractures, hormone replacement use, disease duration and glucorticoid use for low bone mineral density (BMD) in rheumatoid arthritis patients.

Method A cross-sectional study in 177 patients (164 females and 13 males). BMD was assessed in the spine and femoral neck in a DXA Norland XR26. Low BMD was defined as Z score < -1 compared with our normal population. Student's t test, logistic regression, stepwise logistic regression and multiple logistic regression were calculated.

Results See Tables 1 and 2 overleaf.

Conclusions When risk factors for low BMD were analyzed in an age-adjusted and sex-adjusted model, disease duration and glucorticoid use appeared as significant risk factors for low BMD. In a multivariate analysis, disease duration longer than 5 years appears as independently significant for low femoral neck BMD. Related to glucorticoid use, only more than 10 g cummulative dose was independently significant for low BMD both in the spine and femoral neck.

Table 1

Risk factors for low bone mineral density (BMD) in the age-adjusted and sex-adjusted model

Variable	Comparison	Spine, odds ratio (95% confidence interval)	*P*	Femoral neck, odds ratio (95% confidence interval)	*P*
Female	Male	1.43 (0.44–4.68)	0.55	1.35 (0.41–4.47)	0.63
Menopause	Premenopause	0.96 (0.51–1.81)	0.92	1.34 (0.72–2.52)	0.49
Ever smoking	Never smoked	0.88 (0.42–1.89)	0.76	1.19 (0.57–2.51)	0.64
Nonvertebral					
Fracture	No fracture	0.98 (0.28–3.39)	0.98	1.38 (0.42–4.53)	0.59
Ever HRT	Never HRT	0.41 (0.15–1.15)	0.09	0.30 (0.14–1.10)	0.08
Disease duration					
1–5 years	< 1 year	1.51 (0.55–4.06)	0.42	3.07 (0.91–10.34)	0.07
5 years	< 1 year	2.54 (1.06–6.05)	0.04	7.46 (2.47–22.48)	0.0001
Corticosteroids					
Ever	Never	3.19 (1.56–6.54)	0.001	2.07 (1.06–4.02)	0.03
< 7.5 mg daily	Never	2.75 (1.28–5.88)	0.009	1.83 (0.89–3.74)	0.09
7.5 mg daily	Never	4.29 (1.81–10.20)	0.001	2.61 (1.15–5.91)	0.02
< 5 g cummulative	Never	1.51 (0.56–3.99)	0.42	1.04 (0.41–2.67)	0.93
5–10 g cummulative	Never	2.61 (0.99–6.88)	0.05	0.95 (0.34–2.63)	0.92
> 10 g cummulative	Never	5.11 (2.3–11.29	0.0001	4.05 (1.9–8.61)	0.0001

Low BMD, *Z* score < −1 compared with our normal population. HRT, hormone replacement therapy.

Table 2

Multivariable analysis of risk factors for low bone mineral density (BMD)

Variable	Comparison	Spine, odds ratio (95% confidence interval)	*P*	Femoral neck, odds ratio (95% confidence interval)	*P*
Menopause	Premenopause	0.85 (0.42–1.74)	0.66	1.08 (0.52–2.52)	0.83
Ever HRT	Never HRT	0.69 (0.22–2.16)	0.53	0.55 (0.17–1.76)	0.31
Disease duration					
1–5 years	< 1 year	0.95 (0.30–3.07)	0.94	2.31 (0.63–8.52)	0.21
5 years	< 1 year	1.22 (0.41– 3.62)	0.72	4.83 (1.44–16.21)	0.01
Corticosteroids					
Never	Ever	0.87 (0.38–2.03)	0.75	0.69 (0.21–2.23)	0.54
< 5 g cummulative	Never	1.35 (0.37–4.89)	0.65	1.62 (0.45–5.89)	0.46
>10 g cummulative	Never	5.39 (1.95–14.86)	0.0001	3.73 (1.23–11.26)	0.02

Low BMD, *Z* score < −1 compared with our normal population. HRT, hormone replacement therapy.

SLE and Sjögren syndrome

90 (P9.1)

Systemic lupus erythematosus is characterized by faulty B-cell tolerance in the germinal centers

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Studies of human B-cell tolerance have been hampered by the low frequency of antigen-specific autoreactive B cells in healthy subjects as well as by lack of access to secondary lymphoid tissue in autoimmune patients. We have resolved the first problem by studying the fate of a population of human B cells endowed with intrinsic autoreactivity owing to the expression of antibodies encoded by the VH4.34 gene segment (VH4.34 B-cells and VH4.34 antibodies, respectively) [1]. These pathogenic autoantibodies are very abundant in systemic lupus erythematosus (SLE), where they represent 10–40% of serum IgG, correlate with disease activity, renal and central nervous system involvement, and contribute substantially to the anti-dsDNA and anti-lymphocyte antibody repertoire [2–5]. Of note, increased production of VH4.34 antibodies is highly specific for SLE and has not been observed in other autoimmune diseases. Taking advantage of the abundance of VH4.34 B cells in healthy individuals and of the ability of the anti-idiotypic monoclonal antibody 9G4 to specifically recognize VH4.34 B cells, we previously proposed that healthy VH4.34 B cells are negatively selected during the germinal center (GC) reaction [1].

Herein, we have expanded our initial studies to further dissect this observation using tonsil biopsies obtained from patients with SLE ($n = 8$) and rheumatoid arthritis ($n = 3$) as a source of secondary lymphoid tissue. Using both flow cytometry, intracellular calcium analysis and immunocytochemistry, our studies demonstrate that in normal subjects $9G4^+$ autoreactive VH4.34 B cells display an anergic phenotype and undergo strong counter selection in the early phase of the GC

reaction, as indicated by their decline in the transition from the GC founder stage to the centroblast stage and by their absence from proliferative GCs. In contrast, SLE 9G4$^+$ B cells participate in approximately 20% of all productive GC reactions, as substantiated by flow cytometry and histological analyses and as reflected by a 10-fold to 20-fold expansion into the post-GC IgG memory and plasma cell compartments. Remarkably, in patients with RA, 9G4$^+$ B cells behave like healthy 9G4$^+$ B cells and are also strongly counter-selected in the GC. Overall, our results strongly indicate that a critical checkpoint in the maintenance of B-cell tolerance depends on effective negative selection of autoreactive B cells in the GCs and that this checkpoint is specifically defective in SLE. Detailed comparisons of the cellular, biochemical and genetic profiles of the cells targeted at this checkpoint (GC founders) between healthy control and SLE patients should shed considerable light into the mechanisms responsible for disease development, thereby suggesting new therapeutic approaches.

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91 (P9.2)

Interferon-induced gene expression in systemic lupus erythematosus reflects previous exposure to interferon alpha and is associated with increased disease activity and autoreactivity against RNA-binding proteins

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Background Both interferon (IFN) alpha and IFN-γ have been implicated in the pathogenesis of systemic lupus erythematosus (SLE). Recently, microarray screens have demonstrated increased IFN-inducible gene (IFIG) expression in peripheral blood mononuclear cells of patients with active SLE. We investigated the relative roles of IFN-α and IFN-γ in gene expression and disease in SLE patients.

Methods Quantitative real-time PCR was employed to identify IFIGs that are regulated by either IFN-α or IFN-γ. Peripheral blood mononuclear cells from 77 SLE patients were compared with those of 22 disease controls and 28 healthy donors (HD) for expression of three genes preferentially induced by IFN-α (PRKR, IFIT1, IFI44) and three genes preferentially induced by IFN-γ (IRF1, SERPING1, and GBP1) inducible genes. IFN-α and IFN-γ scores were then calculated for all individuals, based on the number of IFIGs overexpressed and the level of increased expression above the mean value for that gene in the HD group. IFNs in plasma were measured by ELISA and assayed for IFIG-inducing activity. Disease activity was assessed using the SLE disease activity index-2000 (SLEDAI-2K) and severity with the number of American College of Rheumatology (ACR) criteria fulfilled and the Systemic Lupus International Cooperating Clinics (SLICC) damage index. Sera were tested for autoantibodies to dsDNA, Sm, ribonucleoprotein, Ro, La, and antiphospholipid antibody (APLA).

Results Two IFN-α-inducible genes (PRKR and IFI44), but no IFN-γ-inducible gene, showed higher expression in SLE than in disease controls ($P = 0.005$ and $P = 0.01$, respectively) or healthy donors ($P = 0.01$ and $P = 0.03$). Moreover, IFN-α scores were higher in SLE patients than in both control groups ($P < 0.01$), with 50% of SLE patients demonstrating a high IFN-α score (defined as ≥ 2). Plasma from all SLE patients contained high levels of IFN-α and some contained IFN-α-gene inducing capacity that was inhibited by anti-IFN-α antibody. Expression of IFN-α-regulated genes correlated with erythrocyte sedimentation rate ($r = 0.33$, $P = 0.003$), serum C3 ($r = -0.3$, $P = 0.008$), C-reactive protein ($r = -0.3$, $P = 0.03$), SLEDAI-2K ($r = 0.22$, $P = 0.05$), and SLICC damage index ($r = 0.33$, $P = 0.003$). It was also associated with a greater number of ACR criteria, Caucasian race, and renal disease. Interestingly, SLE patients had increased prevalence of autoantibodies to Ro ($P = 0.0008$) and to any RNA binding protein (one or more of Sm, ribonucleoprotein, Ro, La; $P = 0.0002$). In contrast, the prevalence of anti-dsDNA or APLA was not associated with IFN gene expression.

Conclusions These data demonstrate that activation of the IFN-α pathway is a characteristic of SLE and that IFN-α is the predominant stimulus for IFIG expression in those patients. IFN-α-inducible genes are candidate biomarkers identifying patients with increased disease activity and define a subgroup of SLE patients with serum autoreactivity against RNA-binding proteins.

92 (P9.3)

An open-label safety and efficacy study of an anti-CD20 antibody (rituximab, Rituxan®) for anti-B-cell therapy in the treatment of systemic lupus erythematosus

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B cells play a central role in the pathogenesis of systemic lupus erythematosus (SLE) [1]. The availability of a highly effective and relatively safe B-cell-depleting drug, rituximab (Rituxan®; Genentech, South San Francisco, CA, USA, and Biogen Idec, San Diego, CA, USA), has permitted us to begin to test the hypothesis that removal of B cells would provide a therapeutic benefit for SLE patients [2]. An initial phase I safety trial was undertaken to ensure that rituximab did not have any unexpected toxicities in this new population. Patients with moderately active SLE (SLE Disease Activity Index > 1) who had failed at least one cytotoxic agent and had current involvement of a major organ system were eligible to enroll in the study. The treatment regimen was the same approved for B-cell lymphoma: a single course of four weekly doses of rituximab intravenously at 375 mg/m^2. Patients received 100 mg methylprednisolone intravenously before each infusion. During the treatment period, they could not be on immunosuppressives, and prednisone doses were kept constant. Patients were followed for 1 year after treatment with repeated laboratory screens, measures of clinical disease activity, and analyses of the subsets of peripheral blood lymphocytes.

To date, 20 patients have been enrolled in the study. The first two patients received lower doses of rituximab, and were found to develop positive human anti-chimeric antibody tests. One patient developed a serious adverse event (hyptotension and bradycardia), which may have been related to the medication. All subsequent patients were scheduled to receive the full therapeutic dose. The results discussed here pertain to the nine patients on whom data were available at the time of abstract submission. All patients tested so far depleted their peripheral blood B cells by at least 95%. Six of eight patients who received the full dose showed an improvement in SLE Disease Activity Index, although four of these 'responders' required the addition of azathioprine or mycophenolate mofetil after 1–6 months. B cells returned to

the peripheral blood as early as 3 months after treatment, although some patients remained depleted after 1 year. Low titer, transient human anti-chimeric antibody positivity was observed in two patients. Rituximab was well tolerated, without infusion-related serious adverse effects. One death occurred in a patient due to severely active SLE.
Serum immunoglobulins, anti-DNA and antinuclear antibody titers did not fall, nor did complement levels change. Patients had antibody titers against pneumococcal polysaccharides and tetanus tested before and after treatment, and then received booster immunizations (Pneumovax and tetanus toxoid) approximately 6 months after treatment. For the four patients in whom results are available, three failed to respond to this antigenic challenge.
This experience suggests that rituximab is potentially effective and reasonably safe for patients with SLE. It remains to be determined how best it should be used (timing and amount of doses, concomitant medications) and whether it will add significantly to the toxicity of immunosuppressive therapies. Randomized, double-blind, placebo-controlled trials are necessary at this point to approach these questions.

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93 (P9.4)

Analysis of IgVH-specific, CXCR4-specific and CXR5-specific mRNA transcripts in individual peripheral blood B cells of patients with primary Sjögren's syndrome and normal healthy subjects

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Background Disturbances in peripheral B-cell homeostasis have been found to be characteristic of primary Sjögren's syndrome (pSS). Abnormal chemokine receptor expression has been proposed to be involved in the pathogenesis of autoimmune diseases.
Objective This study aimed to further delineate disturbances in peripheral $CD27^-$ naive and $CD27^+$ memory B cells and their expression of chemokine receptors in pSS.
Methods Isotype-specific immunoglobulin heavy chain (IgH) transcripts as well as chemokine receptor (CXCR4 and CXCR5) specific mRNA transcripts were analyzed in single-sorted $CD19^+D27^-$ naïve and $CD19^+CD27^+$ memory B cells from pSS patients and normal healthy subjects (NHS).
Results A significantly higher frequency of B cells coexpressing μ, α, and/or γ chain transcripts were found in pSS patients compared with controls (58.0% versus 14.3%, $P < 0.0001$). Contrasting the findings in NHS, peripheral memory B cells in pSS patients were characterized by heavily mutated IgV_H transcripts (mutational frequency, 8.6% versus 4.4%; $P < 0.0001$). Notably, this included significantly enhanced mutational frequencies of Cμ-transcripts (9.6% in pSS versus 2.5% in NHS, $P < 0.0001$). A $CD27^-$ memory-type B-cell subpopulation expressing mutated Cμ-transcripts was exclusively found in pSS patients. Moreover, a significantly enhanced frequency of CXCR4-mRNA-positive $CD27^-$ naive B cells was found in pSS patients when compared with NHS (36.0% versus 18.1%, $P < 0.0001$).
Conclusions Altogether, B-cell hyperactivity and abnormalities in peripheral B-cell memory are characteristic of patients with pSS. Differential expression of chemokine receptors appear to be involved in disturbances of peripheral B-cell homoestasis in pSS.

94 (P9.5)

Lymphoid chemokine expression in Sjogren's syndrome: relationship with the lymphoid organization of the periductal inflammatory aggregates

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Background The inflammatory cellular infiltrate typical of several chronic diseases, including Sjogren syndrome (SS), is often organized in lymphoid-like structures. CXCL13 and CCL21 are lymphoid chemokines critical for physiologic development of secondary lymphoid organs. They have also been implicated in the formation of ectopic lymphoid neogenesis in various experimental and pathological conditions. To define the relationship between the *in situ* production of CXCL13 and CCL21 and lymphoid organization in SS we examined the expression of these chemokines in relation to the degree of B-cell and T-cell segregation, the presence of follicular dendritic cell (FDC) ($CD21^+$) networks and germinal centre (GC) reactions as well as the development of high endothelial venule (HEV) ($PNAd^+$)-like vessels.
Aims The aim of this study was to characterize the organization of neolymphoid tissue in the salivary glands of SS patients and to correlate its development and maturation with the ectopic expression of lymphoid chemokines CXCL13 and CCL21
Methods Periductal foci in 26 SS salivary gland biopsies and nine disease controls with nonspecific sialoadenitis were analysed on the base of a grading score (G1, ≤ 50 cells; G2 > 50 cells; and G3 = G2 + presence of GCs). This was related to follicular organization and maturation assessed in respect to T-cell and B-cell segregation, CD45RO and CD45RA expression ($CD3^+$, $CD20^+$ and UCH10, S130), FDC networks ($CD21^+$) and PNAd (MECA-79+) HEV formation and CXCL13 and CCL21 expression.
Results In SS samples, G1 aggregates showed preponderance of $CD3^+/CD45RO^+$ infiltrating lymphocytes without B/T area segregation, G2 revealed an increasing number of $CD20^+/CD54RA^+$ and a variable degree of organization (54.55% not segregated, 21.21% atypically segregated, 24.24% segregated), while G3 exhibited $CD20^+/CD54RA^+$ majority with the typical segregation of secondary lymphoid follicles. Within G2 and G3 aggregates we identified $CD21^+$ cells clusterized or in a reticular pattern within the GCs. $MECA79^+$ vessels were detected on the edge of the aggregates. CXCL13 expression was seen in 4.07% of G1, 46.16% of G2 and 100% of G3 lymphocytic aggregates. CXCL13 was localized within G2 aggregates, in G3 inside $CD21^+$ GCs and in some infiltrated ducts. CCL21 expression was detected in 2.5% of G1, 17.85% of G2 and 47.62% of G3 aggregates. CCL21 was related with the endothelium of HEV morphology structures and some cells surrounding these structures. In nonspecific sialoadenitis samples we detect no follicle formation or features of secondary lymphoid organ formation.
Conclusions In the salivary gland of patients with SS a true phenomenon of lymphoneogenesis appears to take place, characterized by the formation of mature follicles with GCs, B/T segregation, FDC networks and PNAd expression on HEVs.
CXCL13 and CCL21 expression clearly correlates with the higher grades of organization of the infiltrates. The presence of lymphoid structures within the target organs for the disease and the association of these structure with chemokines acting as regulators of lymphoneogenesis in secondary lymphoid organs, combined with the possible expression of CXCL13 even in the absence of professional FDCs, suggests a key role for these molecules in the pathogenesis, maintenance and evolution of the disease process.

Angiogenesis and apoptosis

95 (P10.1)

P-selectin, an important mediator of angiogenesis in females: the role of estradiol

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Angiogenesis, or new blood vessel formation, is key in vasculoproliferative disorders including rheumatoid arthritis. Here we report that the mediation of angiogenesis by P-selectin, an important member of the selectin adhesion molecule family, is unexpectedly sex dependent. *In vivo* angiogenesis measured in a Matrigel plug, in a sponge granuloma, and in a corneal micropocket was consistently impaired in P-selectin gene deficient mice compared with wild-type mice, but only if the P-selectin-deficient mice were female. Estrogen appeared to be one significant mediator of P-selectin sensitivity. *In vitro* the chemotactic activity of soluble P-selectin for human dermal microvascular endothelial cells was augmented by 17β-estradiol (E_2). Conversely, an antibody blockade of P-selectin reduced E_2-induced sprout formation in female mouse corneal endothelial cell morphogenesis assays, inhibited E_2-induced endothelial cell signaling via the Src and mitogen-activated protein kinase pathways, and decreased E_2-induced secretion of angiogenic basic fibroblast growth factor. In addition to its classical roles in leucocyte extravasation, P-selectin may also contribute to a variety of inflammatory diseases as a sex-restricted angiogenic intermediary.

96 (P10.2)

Synovial cadherin-11

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Cadherin-11 is a homophilic adhesion molecule that is expressed on fibroblast-like synoviocytes. To investigate a role for cadherin-11 in modulating synovial function, we have examined the synovial architecture and inflammatory responses in cadherin-11 mutant mice. We find these mice display a hypoplastic synovial lining with decreased cellular condensation and reduced extracellular matrix deposition. When challenged with arthritogenic serum in the KRN anti-GPI antibody-induced arthritis model in mice, cadherin-11-deficient animals display resistance to arthritis development. In animals that do develop detectable swelling and inflammation, there is a noted absence of cartilage damage, compared with wild-type animals with arthritis. Moreover, the architecture of the synovial response to inflammation is disorganized. In contrast to wild-type arthritic mice in which the synovial lining undergoes marked hyperplasia, the cadherin-11-deficient synovial reaction lacks a clearly detectable lining layer and shows a disorganized random inflammatory reaction. These results support the concept that fibroblast-like synoviocytes are direct participants in the highly orchestrated synovial reaction that occurs in inflammatory arthritis. Furthermore, these results reveal a role for synovial cadherin-11 in regulating synovial fibroblast function both in the healthy state and in the pathologic context of inflammatory arthritis.

97 (P10.3)

Effects of hypoxia on protein and gene expression in fibroblast-like synoviocytes

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Background The synovial microenvironment in rheumatoid arthritis (RA) is often hypoxic with evidence of anaerobic metabolism. This results from an imbalance between high metabolic demands and impaired tissue perfusion due to microvascular damage. Cellular responses to hypoxia are mediated by the transcription factor HIF-1α, which is exquisitely and rapidly controlled by cellular oxygen tension. HIF-1α has been shown to play a key role in promoting tumor angiogenesis, and was recently shown to be critically important in inflammatory responses.
Methods To better define the effects of hypoxia in the synovial microenvironment, we studied the response of fibroblast-like synoviocytes (FLS) to hypoxic stimulation using the gene expression microarray, quantitative RT-PCR, and matrix-assisted laser desorption-time of flight mass spectrometry. The expression of HIF-1α in fresh RA synovial tissue explants was evaluated under both normoxic and hypoxic conditions. In addition, FLS were infected by an adenoviral vector carrying a modified HIF-1α gene with the oxygen degradation domain removed. This vector has been shown to induce normoxic expression of genes with hypoxia responsive elements.
Results Under normoxic conditions, HIF-1α expression in fresh samples of RA synovium was patchy and generally confined to the nuclei of cells in hyperplastic lining layers. Under conditions of 1% O_2 or $CoCl_2$, HIF-1α expression was markedly increased in FLS and in the lining cells of synovial tissue explants. Interestingly, while hypoxia induced the stabilization of HIF-1α protein by preventing its degradation, it also resulted in a significant downregulation of HIF-1α gene expression. Of the approximately 80 genes known to be directly regulated by HIF-1α, 75% were found to be upregulated by hypoxia in FLS. This included angiogenic mediators such as vascular endothelial growth factor, angiopoietins, and leptin, apoptosis mediators such as BNIP3, glycolysis-related enzymes such as G6PI, and the chemokine CXCL12 and its receptors CXCR4. Regulation of CXCL12 by HIF-1α was confirmed by its normoxic induction in adenoviral infected FLS lines. In addition, a spectrum of novel genes and proteins not known to be regulated by HIF-1α were shown to be induced in FLS by hypoxic stimulation.
Conclusions Hypoxic conditions in RA synovium promote persistence by inducing angiogenesis, enhancing FLS survival, and enhancing lymphocyte recruitment. Hypoxic induction of G6PI may promote the development of anti-G6PI autoantibodies.

98 (P10.4)

Long-term exposure of rheumatoid arthritis synovial fibroblasts to tumor necrosis factor alpha inhibits FasL-mediated apoptosis through activation of NF-κB and upregulation of the small ubiquitin-like modifier SUMO-1

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Objective There is accumulating evidence that rheumatoid arthritis synovial fibroblasts (RA-SF) are resistant to FasL-induced apoptosis despite the abundant expression of Fas. Tumor necrosis factor alpha (TNF-α) has been suggested to contribute to this process mainly through the transient activation of transcription factors such as NF-κB. However, in addition to short-term induction of transcriptional regula-

tors, long-term activation of RA-SF has gained increasing interest. In this context the small ubiquitin-like modifier SUMO-1 appears to be of importance, and some data indicate that increased levels of SUMO-1 are linked to the resistance of RA-SF against programmed cell death. However, little is known about the regulation of SUMO-1 in fibroblast-like cells. Here, we investigated the effects of long-term stimulation of RA-SF with TNF-α on the activation of NF-κB, the expression of SUMO-1, and on spontaneous as well as FasL-induced cell death.

Methods Synovial tissues were obtained from patients with rheumatoid arthritis at joint replacement surgery, and synovial fibroblasts were isolated by enzymatic digestion. Long-term effects of TNF-α were analyzed by stimulation of RA-SF with 10 or 100 ng/ml TNF-α for 24 hours. Nuclear binding of NF-κB was assessed by electrophoretic mobility shift assay. The expression of SUMO-1 in TNF-α-stimulated and unstimulated RA-SF was determined by quantitative real-time PCR and western blot. To induce apoptosis, TNF-α pretreated and untreated RA-SF were stimulated with recombinant human FasL (100 ng/ml) for 16 hours. Apoptosis was measured by a histone fragmentation assay (Cell Death ELISA; Roche Diagnostics, Mannheim, Germany) and confirmed by FACS analysis with intercellular TUNEL staining (Apo-BRDU™ Kit; BD Biosciences, Heidelberg, Germany).

Results Treatment of RA-SF with TNF-α over 24 hours did not induce cell death but slightly reduced the rate of spontaneous apoptosis. More significantly, long-term exposure of RA-SF to TNF-α clearly prevented the induction of apoptosis by recombinant human FasL in a dose-dependent manner. This was accompanied not only by a sustained activation of NF-κB, but also by a significant increase in the expression of SUMO-1. The induction of SUMO-1 by TNF-α was dose dependent and seen both at the mRNA and the protein level.

Conclusions The data suggest that long-term exposure of RA-SF to TNF-α inhibits FasL-induced apoptosis not only through sustained activation of NF-κB, but also through upregulation of the small ubiquitin-like modifier SUMO-1. Although SUMO-1 has been demonstrated to be elevated in RA-SF in the absence of continuous stimulation with inflammatory cytokines and to be part of the stable activation of RA-SF, TNF-α in the inflamed synovium may enhance further the expression of SUMO-1 and, thus, contribute to the resistance of RA-SF against apoptosis.

99 (P10.5)

Tumor necrosis factor alpha modulates fibroblast-like synoviocyte expression of lymphangiogenesis-associated vascular endothelial growth factor C

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Purpose Vascular endothelial growth factor C (VEGF-C) is a member of the platelet-derived growth factor/vascular endothelial growth factor family of growth factors. Functionally, VEGF-C has been characterized as both a lymphangiogenic and angiogenic factor and can induce chemotaxis of macrophages. The exact mechanism by which the angiogenic, lymphangiogenic and cell recruitment processes are induced and maintained in the rheumatoid synovium of patients with rheumatoid arthritis (RA) is currently not known. We hypothesized that VEGF-C may be involved in RA and in this study proceeded to characterize its regulation in synoviocytes and tissue from RA and osteoarthritis (OA) patients.

Methods Protein lysates were prepared from synovial tissue isolated from seven RA and six OA patients undergoing arthroplasty. Fibroblast-like synoviocytes (FLS) were grown out of synovial tissue samples and used at passages 2–5. Western blotting was employed to examine the VEGF-C isoforms. RT-PCR was used to examine VEGF-C receptors.

Results Western blots revealed an increase in the processed forms of VEGF-C in protein lysates prepared from synovial tissue of RA compared with OA patients. FLS produced substantial amounts of VEGF-C, the majority of which was secreted as the partially processed 29-kDa and 31-kDa forms that have been reported to bind to the lymphangiogenesis-associated VEGFR-3. These secreted forms were increased about 1.6-fold ($P = 0.02$) in RA FLS compared with OA FLS. The addition of 10 μg/ml tumor necrosis factor alpha (TNF-α) to the culture media increased the secreted VEGF-C forms an average of 1.4-fold in RA lines and 1.9-fold ($P = 0.002$) in OA lines. Additionally, mRNA levels for neuropilin-2, a receptor for VEGF-C and class 3 semaphorins that has been shown to be necessary for the formation of small lymphatic capillaries, were increased approximately 20-fold in both OA and RA lines 24 hours after TNF-α addition.

Conclusions This is the first study to demonstrate that TNF-α-driven FLS may modulate synovial inflammation and joint destruction by paracrine and/or autocrine mechanisms involved in lymphangiogenesis.

Students – Arthritis Society/European League Against Rheumatism

100 (P11.1)

A noninvasive magnetic resonance imaging-based method for assessing patellofemoral joint kinematics detects differences between healthy and symptomatic knees

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Patellofemoral joint pain is one of the most common pathologies observed in orthopedic clinics. It is widely held that abnormal joint mechanics play a causative role in the development of anterior knee pain and that chronic patellofemoral joint pain may lead to cartilage degeneration, arthritis and permanent disability [1]. However, the relationship between joint kinematics and clinical symptoms remains unclear. We applied a new magnetic resonance imaging (MRI)-based method to assess three-dimensional patellar tracking during loaded knee flexion to determine whether anterior knee pain is associated with abnormal joint kinematics.

The symptomatic and matched knees of 60 subjects (20 cases with anterior knee pain and clinical evidence of patellar malalignment; 20 cases with anterior knee pain and no clinical evidence of patellar malalignment, and 20 controls matched for age, gender and physical activity level) were imaged through a range of loaded knee flexion using a validated MRI-based method [2]. Using this novel evaluation tool, the motion of the patella relative to the femur was determined as a function of knee flexion angle. Group values through a common range of knee flexion were compared using analysis of variance.

In the control group, the normal pattern of patellar motion is increasing medial rotation, tilt and shift as the patella approaches the trochlear groove during loaded knee flexion (from 19° to 28°). In the group with pain and clinical signs of malalignment, the patella is translated 2.6 mm more laterally and rotated 1.3° more medially ($P < 0.001$). Statistically, differences in patellar kinematics are observed between the control group and the group with pain and no clinical evidence of malalignment; however, the magnitude of the differences are all within the range of the accuracy and repeatability of the technique with the exception of the decreased distance between the patella and femur ($P < 0.001$).

This MRI-based method detects a normal pattern of patellar kinematics that is consistent with previous reports [3] and detects abnormal kinematics in subgroups of individuals with anterior knee pain. In the cases with pain and clinical evidence of patellar malalignment, the increased lateral shift and medial rotation is consistent with weakness of the vastus medialis oblique muscle, which has been linked with patellofemoral joint pain previously. Our results do not support the notion that increased lateral tilt is associated with patellofemoral joint pain. In the cases with pain and no clinical evidence of patellar malalignment, the closer positioning of the patella relative to the femur may reflect a decrease in cartilage thickness. Of note is that in most cases the pattern of patellar motion was similar to that for the controls. Longitudinal studies using this novel MRI-based method will aid in determining the link between knee pain and joint mechanics.

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101 (P11.2)

Perceived need for workplace accommodation and labour force participation in Canadian adults with arthritis disability

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Purpose Although reduced labour force participation is often a consequence of physical disability, little is known about the role of workplace accommodation. This study uses a conceptual model based on the World Health Organization International Classification of Functioning, Disability, and Health and hypothesizes perceived need for workplace accommodation as a mediating variable between activity limitation and not in labour force.

Methods Data from the Canadian Health and Activity Limitation Survey were used. Working-age participants (25–64 years) with arthritis disability were included. Employment status was dichotomized into 0 = in labour force (employed and unemployed), 1 = not in labour force. Two latent constructs (lower body and upper body disability) were used to represent 12 categorical physical disability indicators (e.g. difficulty in walking) and one latent construct was derived for eight workplace accommodation indicators (e.g. lack of accessible workstation, elevator or flexible hours if needed). Personal variables (age, sex, education, and occupation) were also incorporated into the model. MPLUS was used to perform the categorical factor analysis and standard error of the mean analyses.

Results Physical activity limitations affected labour force participation both directly and indirectly through perceived need for workplace accommodation. As people's activity limitations became severe they were more likely to perceive the need for workplace accommodation, and in turn, this lead to reduced labour force participation. Lower body activity limitations had more impact on labour force than upper body activity limitation. Older age, female gender, and low education were also associated with reduced labour force participation.

Conclusion Most of the effect of arthritis-associated physical activity limitations and all on labour force participation is mediated by perceived workplace accommodation, which underscores the importance of workplace accommodation provision.

102 (P11.3)

Magnetic resonance imaging, X-ray and dual X-ray absorptiometry techniques for assessment and monitoring of knee osteoarthritis

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Aims Research projects completed and ongoing by members of our research team include: (i) validation of quantitative cartilage measurements using a 1 T extremity magnetic resonance imaging (MRI) scanner; (ii) radiographic evaluation of soft-tissue structures – a comparison between 1 T and 1.5 T MRI scanners; (iii) short-term and long-term reproducibility of computer-determined medial minimum joint space width (mJSW) of the knee using a fixed-flexion X-ray protocol; (iv) determination of 'normal' values of minimum medial joint space width of the knee, bone density using dual X-ray absorptiometry around the knee joint and cartilage volume and thickness in men and women per decade between the ages of 20 and 69 years; (v) determination of values of medial mJSW of the knee, bone density around the knee joint and cartilage volume and thickness in men and women with varying degrees of knee osteoarthritis (OA); (vi) development of a longitudinal database of patients with OA of the knee, documenting pain, physical function, quality of life, history, medication use, family history, physical examination, blood tests, plain film radiographs and MRI of the knee using a 1 T extremity MRI scanner; (vii) development of a more fully automated technique for cartilage segmentation of three-dimensional (3D) MRI data; and (viii) quantification of 3D trabecular bone structure using high-resolution MRI.

Methods Healthy volunteers and patients with knee OA referred for assessment to a rheumatologist are invited to participate in the various studies. All patients are asked to complete preset questionnaires. Radiographs of the knee are performed postero-anteriorly in a standardized fixed-flexion position by trained technicians. Radiographs are subsequently digitized and analyzed for mJSW using an automated computer algorithm. A 1 T extremity MRI scanner is used for imaging each individual's knee. Sequences include a saggital T_1-weighted, 3D spoiled gradient echo with fat saturation, a saggital T_1-weighted fast spin echo (FSE), a coronal T_2-weighted FSE and an axial FSE inversion recovery sequence.

Results Quantitative cartilage measurements of normal and OA subjects' knees were obtained using a validated segmentation technique on both 1 T and 1.5 T MRI images. The 1 T extremity MRI scanner has been found to be reliable and of comparable precision in comparison with a 1.5 T whole-body scanner.

The use of a fixed-flexion position and a computer algorithm to determine medial mJSW of the knee in patients having normal or osteoarthritic knees has been found to be reliable in both short-term and long-term studies. Forty-seven pairs of knee radiographs were assessed in the short-term study while 14 pairs in a long-term study were assessed. Reliability for both studies was measured by means of intraclass correlation coefficient and found to be 0.95–0.99 for both healthy subjects and those with OA.

A cross-sectional study of 45 patients referred for assessment of knee pain is being completed. Data analyses will include comparison of pain scales, history and physical examination with MRI findings in the knee.

Conclusions Quantitative cartilage measurements using a 1 T office-based extremity MRI scanner have been found to be reliable and of comparable precision with those obtained using a 1.5 T whole-body MRI scanner. The use of a fixed-flexion radiographic technique for assessing mJSW has been found to be reliable in a long-term reproducibility study at our center. Early diagnosis and prompt assessments of patients with OA can be accessible using an office-based extremity MRI scanner and could prove to be a valuable clinical tool with the development of disease-modifying agents for the treatment of OA.

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