1. Preclinical changes in immune-mediated inflammatory disease

**A1.1** ANTI-CITRULLINATED PROTEIN ANTIBODY SPECIFIC Fc GLYCOsyLATION PATTERNS IN ARTHRAlGIA PATIENTS

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**Background/Objectives** During rheumatoid arthritis, anti-citrullinated protein antibodies (ACPA) exhibit a specific, pro-inflammatory Fc glycosylation profile that is characterised by a low content of galactose and galactosic acid residues. The absence of these sugars from the Fc-linked core glycan could influence the biological activity of ACPA during disease. As ACPA can be detected in sera several years before disease development, we hypothesised that a change in ACPA Fc-glycosylation might precede the onset of arthritis.

**Methods** Serum samples (n = 300) from patients with ACPA positive arthralgia (n = 184) were obtained at various time points. In this cohort, 96 patients developed arthritis after an average duration of 14.7 months of arthralgia. At the time of the onset of arthritis, patients were defined as having rheumatoid arthritis (RA, n = 51) based on the 1987 ACR criteria for RA, or undifferentiated arthritis (UA, n = 45). ACPA were isolated from serum samples by affinity purification, using cyclic citrullinated peptides as antigen. Purified ACPA-IgG and total serum IgG were digested with trypsin and purified protein antibodies (ACPA) exhibit a specific, pro-inflammatory Fc glycosylation pattern that is characterised by a low content of galactose and galactosic acid residues. The absence of these sugars from the Fc-linked core glycan could influence the biological activity of ACPA during disease. As ACPA can be detected in sera several years before disease development, we hypothesised that a change in ACPA Fc-glycosylation might precede the onset of arthritis.

**Results** No significant change in Fc-glycosylation patterns was found between ACPA-specific and total serum IgG at the patients’ first presentation with arthralgia (baseline). However, at diagnosis of arthritis, RA patients but not patients with UA exhibited increased hypogalactosylation of the ACPA and total IgG Fc fragments compared to healthy donors. The decrease of ACPA galactosylation levels at the Fc-tail occurred 6 months before diagnosis and was significantly more pronounced at 3 months before diagnosis compared to total IgG.

**Conclusions** A decrease in Fc-galactosylation levels of ACPA occurred around 6 months prior to RA onset. Of interest, this ACPA hypogalactosylation was more pronounced than that of total IgG1, indicating that a more pro-inflammatory Fc-glycosylation pattern could be one mechanism driving inflammation in RA.

**A1.2** FIBROBLASTS INFLUENCE LYMPHOCYTE RECRUITMENT AND MIGRATION DURING RESOLVING AND PERSISTENT ARTHRITIS

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**Background and Objectives** Fibroblasts actively regulate the recruitment of leukocytes by endothelial cells (EC), acting in a pro- or anti-inflammatory manner depending on their site of origin. The phenotype of the fibroblast may be a critical determinant of whether leukocyte recruitment, and therefore inflammation, resolves or persists. Here we examined how synovial fibroblasts from different stages of arthritis influenced the recruitment of peripheral blood lymphocytes (PBL) and their onward migration.

**Materials and Methods** Fibroblasts were isolated from patients with resolving or persistent arthritis. Rheumatoid arthritis (RA) cohorts were categorised based on the stage of the disease at the time of sample collection: very early; newly presented but established or long established undergoing replacement surgery. Two forms of co-cultures were developed: (1) To assess effects on recruitment, EC and fibroblasts were cultured on opposite sides of porous filters and incorporated into a novel flow chamber. PBL were perfused and observed as they bind to the EC surface. (2) To examine effects on migration, EC monolayers were formed on a filter above a collagen gel in which fibroblasts were incorporated. PBL migration through the construct and their location within the gel were assessed. Conditioned media from co-cultures were collected and analysed by Luminox.

**Results** Fibroblasts from patients with RA increased the ability of EC to support PBL recruitment from flow in a disease duration-specific manner, with binding increasing from very early < established < replacement. However, levels of binding to very early RA co-cultures were similar to those observed when fibroblasts from non-inflamed or resolving tissue were incorporated. In the multi-cellular gel model, all fibroblasts, expected those from non-inflamed tissue, promoted PBL transendothelial migration but had no effect on entry into the gel construct. Interestingly, a greater proportion of PBL migrated into the lower half of the gel when fibroblasts from patients with very early and established RA were incorporated. Elevated levels of IL-6, IL-1β, IL-8, Groα and IP-10 were detected in the supernatants from RA co-cultures compared to resolving co-cultures. Resolving fibroblasts dramatically reduced the secretion of these soluble mediators by EC, suggesting they potentially have a suppressive effect.

**Conclusions** Collectively these initial data indicate that changes in the ability of fibroblasts to influence endothelial and lymphocyte behaviours may occur very early in the development of RA. Moreover, some of these changes are distinct from the phenotype exhibited by fibroblasts taken from non-inflamed tissue and acutely resolving arthritis.

**A1.3** CITRULLINATION IN HEALTHY AND INFLAMED LUNG TISSUE AS A PRIMING SITE FOR AUTOIMMUNITY IN RA

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**Background** Anti-citrullinated peptide/protein antibodies (ACPAs) are the key pathogenic autoantibodies in rheumatoid arthritis (RA). Because smoking is a risk factor for RA and ACPA often appear years before the onset of disease, it has been proposed that the lung may be a site for priming the ACPA response. Previous studies using immunohistochemistry suggested that smoking upregulates the expression of PAD2 and PAD4 with the resultant increased expression of citrullinated proteins. However these studies are limited by the availability of healthy lung tissue. In this study we used lung tissue taken at a distance from the primary tumour in lobectomy specimens and antibody reactivity to PAD2 and PAD4 and to two important precursor antigens in RA, alphaelastase and fibrinogen, was defined by immunoblotting to ensure specificity.

**Methods** Lobectomy specimens from 40 subjects undergoing surgery for tumours or bronchiectasis (10 never smokers, 10 smokers without airflow limitation, 10 COPD ex-smokers and 10 COPD current smokers) were carefully dissected to remove a sample of uninvolved lung. The tissue samples were examined by immunoblotting with an anti-modified citrulline (AMC) antibody and scored for the
level of citrullination with a semi-quantitative score from 0–3 by two blinded observers. The presence of PAD2, PAD4, alpha-enolase and fibrinogen was also determined by immunoblotting and scored. Recombinant proteins were used as positive controls and blots with secondary antibodies only were carried out to exclude non-specific cross-reactivity.

Results Citrullinated proteins were detected in 37 out of the 40 lung tissue samples, including 9 out of 10 never smokers. The number of bands and intensity was slightly increased in the COPD smokers (mean score 1.7), followed by COPD ex-smokers (1.4), smokers (1.3) and never smokers without airflow limitation (1.1). PAD2 was detected in all samples, with the band intensity scores correlating roughly with those seen for citrullinated proteins i.e. highest amongst the COPD smokers (mean score 1.9) and lowest amongst never smokers (mean score 1.1). PAD4 and the RA antigens alpha-enolase and fibrinogen were observed in all lung tissue in comparable amounts regardless of disease and smoking status. There was also evidence of citrullination of alpha-enolase provided by co-migration of a ~50KD band recognised by AMC and an anti-enolase antibody, and the demonstration that alpha-enolase in lung tissue ran at several isoelectric spots by 2D electrophoresis.

Conclusions We have shown that there is widespread citrullination of proteins in lung tissue from never smokers, and that there is a modest increase with smoking and COPD. This pattern of expression corresponds to that of PAD2. There is also expression of at least two major RA autoantigens and of PAD4. This supports the hypothesis that the lung is a site for priming the ACPA response, which is enhanced by smoking and COPD.

A1.4 EARLY SIGNS OF SUBCLINICAL INFLAMMATION AND LOCAL ANTIBODY PRODUCTION IN EARLY RHEUMATOID LUNGS

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Objective To investigate if lung changes are present in RA patients early in the disease process and to address the contribution of these changes to disease initiation.

Patients and Methods 21 RA patients with symptom duration less than 1 year at the time of diagnosis and naive to DMARD treatment and 8 healthy individuals were subjected to bronchoscopy and mucosal large bronchial biopsies were retrieved. Histological analysis for identification of inducible bronchia associated lymphoid tissues (iBALT), PAD enzymes, CD3, HLA-DQ and HLA-DR expression were performed. Presence of citrullinated targets were detected by immunohistochemistry using biotinylated ACPA isolated from synovial fluid of RA patients. Presence of ACPA and ACPA fine specificities were tested by ELISA in the serum and BAL of RA patients. Mass spectrometry was used for identification of citrullinated epitopes in 6 of the lung biopsies and additional 8 synovial RA biopsies.

Results iBALT formation and higher expression of CD3, HLA-DQ, HLA-DR and citrullinated targets was observed in bronchial biopsies of ACPA positive RA. A majority of serum ACPA positive RA patients subjected to lung bronchoscopy had detectable levels of ACPA in the BAL fluids both IgA and IgG. IgG from BAL fluids of ACPA-positive patients showed a higher ACPA reactivity as compared to serum IgG from the same patients. Mass spectrometry identified 5 proteins in the synovium (in total 8 sites) and 4 in the lungs (in total 6 sites) containing citrullinated residues. Two vimentin derived citrullinated peptides were present in a majority of both synovial and lung biopsies with slightly higher citrullinated/unmodified peptides ratios in the smokers as compared to non-smokers.

Conclusions Lung subclinical inflammation is present already at the earliest visit to a rheumatology specialist early after disease onset in ACPA + RA patients. These findings suggest that the lungs might be the primary local initiation sites of the anti-citrulline response in RA.

A1.5 EXPLORING THE ROLE OF THE LYMPH NODE MICROENVIRONMENT IN HEALTH AND DISEASE

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Background and Objective Rheumatoid arthritis (RA) is an immune-mediated inflammatory disease of unknown etiology. Changes in the lymph nodes could precede those in the synovial joints. Recent studies reported the importance of lymph node stromal cells (LNSCs) in lymphoid homeostasis, peripheral tolerance and the adaptive immune response. Therefore, we characterised the functional capacities of LNSCs in human lymph nodes during health and different phases of RA.

Materials and Methods Individuals with arthralgia but without any evidence of arthritis upon physical examination who were positive for IgM rheumatoid factor and/or anti-citrullinated protein antibodies were included (n = 12; RA risk group). In addition, we included patients with early arthritis (disease duration <1 year, n = 6), RA (n = 15) and healthy controls (n = 8). All study subjects underwent ultrasound-guided inguinal lymph node biopsy. LNSCs were isolated and cultured from freshly collected lymph node needle biopsies. LNSCs originating from different culture passages were studied to investigate the effects of in vitro culture on the expression level of stromal cell associated genes, including VCAM-1, CollVa and IL-6. In addition, we analysed the expression of Def1 and Aire, transcriptional regulators involved in peripheral tolerance. Functional capacities of LNSCs were studied by analysing STAT-1 and MxA mRNA induction and interleukin (IL)-6 and IL-8 production after TLR-3 triggering by PolyI:C.

Results Cells with a fibroblast-like morphology started to grow out from the stromal part of lymph node biopsies within a few weeks of culture. Passage 0 consisted of a mixture of adherent cells, resulting in a lower expression of the measured stromal genes. From passage 1 gene expression levels for VCAM-1, CollVa and IL-6 increased and stabilised. All LNSCs cell lines expressed Def1 while the expression of Aire was only detected at very low levels. LNSCs were sensitive for TLR-3 ligation by PolyI:C resulting in upregulated STAT-1 and Mxa, downregulated Def1 and high levels of IL-6 and IL-8. Chemokines CCL19 and CCL20 were only expressed after stimulation with PolyI:C. There was a high variability between donors and interim analysis showed no clear differences between LNSCs cultured from healthy individuals, RA risk or RA patients.

Conclusions We developed a culture system for human LNSCs to facilitate research on the role of the lymph node microenvironment in the pathogenesis of RA. Cultured human LNSCs express typical stromal cell markers and are responsive for TLR-3 triggering. Interestingly, the LNSCs express the transcriptional regulator Def1 which may indicate peripheral tissue antigen expression by LNSCs.