autoreactive B cell responses. Our aim was to compare basal activity and induced phosphorylation of AKT, ERK, p38 MAPK and CREB after stimulation of B cells from RA patients and healthy individuals via BCR and/or TLR9.

Materials and Methods Blood samples were collected from healthy donors and RA patients having moderate (DAS28 3.2 < 5.1) and active (DAS28 > 5.1) disease. B cells were stimulated with anti-Ig $(Fab')_2$ and/or CpG ODN. Naive and memory B cells were identified by anti-CD20-A647 and anti-CD27-PE Phosphorylation level of AKT, ERK, p38 and CREB was detected before and after the stimuli by specific phospho-antibodies using multiparameter phospho-flow analysis. Results were evaluated by the FlowJo software.

Results The basal level of phosphorylation of signalling molecules was significantly higher in RA patients as compared to healthy donors. The induced level of phosphorylation was also higher in RA samples in most of cases, CpG stimulated memory B cells from patients with active disease have shown the highest values. In anti-Ig plus CpG ODN stimulated samples phosphorylation of all molecules was significantly higher in both naïve and memory RA B cells as compared to healthy controls. However, when compared to unstimulated cells, the increment of phosphorylation in the stimulated cells was the same or lower in RA samples.

Conclusions We have shown differences in the activation state of AKT, ERK, p38 and CREB in B cells from healthy individuals and RA patients. The higher basal phosphorylation level indicates the activated state of RA B cells. The lower capability of activation-induced phosphorylation may be a result of lower responsiveness of RA B cells. The analysis of phosphorylation signature in RA B-cells may provide new information to a better understanding of the disease.

A5.24 NEUTROPHIL GRANULOCYTES RESPOND TO SURFACE-BOUND IMMUNE COMPLEXES CONTAINING ANTI-TYPE II COLLAGEN ANTIBODIES FROM RA PATIENTS

doi:10.1136/annrheumdis-2013-203219.24

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Background and Objectives We have earlier shown that surface-bound immune complexes containing anti-type II collagen antibodies (anti-CII IC) from rheumatoid arthritis (RA) patients and anti-CII IC stimulate monocyte proinflammatory cytokine production, associated with an acute onset RA phenotype. Anti-CII IC in joint cartilage are exposed to cells in the synovial fluid (SF). Neutrophil granulocytes are the major cell type in SF, where they co-localise with mononuclear cells (MNC). The objective was to investigate whether also granulocytes respond to anti-CII IC, and whether such a response was dependent on interaction with other cells in SF.

Materials and Methods An anti-CII RA serum together with human native collagen (CII) was used to create surface-bound anti-CII IC. Heparinised blood from 8 healthy donors was separated into neutrophil granulocytes (>95% purity) and MNC. For each donor, the granulocyte cell fractions as well as co-cultures (granulocytes + MNC) (0.5 × 10E6/ml of each cell fraction) was cultured on anti-CII IC as well as on negative control IC prepared with normal human serum on CII and in a positive control IC system with purified IgG coated onto plastic. After 18 hours, cells were harvested for the measurement of CD11b, CD66b, CD16 and CD32 on granulocytes by flow cytometry, and supernatant levels of TNF and IL-8 was measured by ELISA.

Results In granulocyte cultures both anti-CII IC and control IC induced significant up-regulation of CD11b and CD66b, and significant down-regulation of CD16 and CD32. When the granulocytes were co-cultured with MNC, there was a significant increase in CD11b up-regulation and CD16 down-regulation than granulocytes, with no effect on CD32 and CD66b. In the co-culture system, the anti-CII IC-induced production of IL-8 was significantly increased, but no such difference was noted for TNF. Isolated granulocyte fractions produced very low levels of TNF and IL-8 after IC stimulation.

Conclusions Isolated granulocytes respond to RA anti-CII IC in a model system mimicking IC in RA cartilage. The granulocyte responses depend on interaction with MNC.

Our anti-CII dependent RA phenotype is a human counterpart to collagen antibody-induced arthritis. Strong granulocyte reactivity to anti-CII IC might therefore be related to the Ncf1 gene involved in NADPH activity important in collagen-induced arthritis models.

A5.25 PROTEOMIC ANALYSIS OF ANTI-CCP IMMUNOGLOBULINS FOR THE IDENTIFICATION OF RHEUMATOID ARTHRITIS PATIENTS THAT REQUIRE EARLY AGGRESSIVE TREATMENT REGIMENS

doi:10.1136/annrheumdis-2013-203219.25

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Background and Objectives Antibodies against citrullinated peptides (ACPA) are a hallmark of rheumatoid arthritis (RA) patients and are associated with poor outcome. ACPA are usually detected using the anti-CCP test, identifying antibodies against a broad spectrum of citrullinated antigens. Most citrullinated antigens however appear to be bystander antigens that are not thought to be involved in the disease process. While controversy remains over the identity of the pathogenic antigen, it has been hypothesised that the fine-specificity of the ACPA response in the patient harbours prognostic properties. For this purpose sera will be studied from early RA patients using a novel technique that might reveal differences in that fine-specificity of the ACPA response.

Materials and Methods We recently showed that the antibody response to a particular antigen results in rearranged immunoglobulin segments that are shared between individuals exposed to that antigen. In patients with paraneoplastic neurological syndromes, such sequences were found after proteomics analysis of affinity enriched immunoglobulins. In the current study, ACPA positive RA sera were drawn from 58 patients participating in the tREACH-study. This is a study with a protocolised treatment regime for patients with early RA aimed at obtaining low-disease activity (DAS < 2.4). Poor outcome was defined as the need for treatment with anti-TNF to control disease activity. Sera were affinity enriched using CCP2 ELISA plates, and the specific IgG was analysed by LC-MS.

Results Up to 1 µg of CCP specific IgG was obtained from 180 µl of serum. The LC-MS data was analysed for correlations between ACPA derived peptides and the need for treatment with anti-TNF to control disease activity. However, no such correlations were found in excess of the false discovery rate in this dataset. In addition to ACPA, we also affinity purified rheumatoid factors from sera as a control. These preparations could be distinguished from the ACPA, suggesting our method performed appropriately.

Conclusions Our experiments could not show a significant difference between anti-CCP antibodies in early RA sera from patients with different disease outcome. While this could indicate that anti-CCP fine-specificities are not associated with disease outcome,

additional experiments are needed to interpret these results. In future work we will compare IgG with known affinity to subclasses of CCP antigens to verify that our technique distinguishes these more subtle differences in epitope specificity. In addition, it will be investigated whether fine specificity may affect the progression to arthritis in ACPA+ arthralgia patients.

A5.26 REGULATION OF EXPRESSION AND FUNCTION OF NEGATIVE IMMUNOMODULATORY RECEPTORS IN B-CELLS: IMPLICATIONS FOR THE PATHOGENESIS OF SYSTEMIC LUPUS ERYTHEMATOSUS

doi:10.1136/annrheumdis-2013-203219.26

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Background and Objectives Fine tuning of B-cell activation and differentiation depends on convergent signals from the B-cell receptor (BCR), costimulatory/coinhibitory membrane receptors, and Toll-like receptors. We sought to examine the expression and function of the coinhibitory receptors programmed death-1 (PD-1), PD-1 ligand-1 (PD-L1), B and T lymphocyte attenuator (BTLA) in B-cells from healthy donors, and from patients with systemic lupus erythematosus (SLE), the prototype of systemic autoimmune disease characterised by activated B-cells and production of high-titer autoantibodies by long-lived plasma cells.

Materials and Methods Peripheral blood $CD19^+$ B cells were purified from healthy donors (n = 11) and active SLE patients (n = 15; SLE disease activity index 8.3 ± 2.7 [mean ± SEM]). PD-1, PD-L1, and BTLA were examined by flow cytometry in naïve ($CD19^+CD27^-$), memory/transitional ($CD19^+CD27^+$), and plasma B-cells ($CD19^+CD27^{hi}$) at baseline and following stimulation. Activation, differentiation, and proliferation (CFSE dilution) of B-cells were examined in the presence or absence of the BTLA ligand, HVEM. Western blot was used to assess the phosphorylation of intracellular kinases.

Results In healthy donors, the coinhibitory receptors PD-1 and PD-L1 were significantly upregulated on circulating plasma-cells compared to transitional/memory and naïve B-cells (PD-1: 36 ± 7%, 14 ± 3%, 2.0 ± 0.5%; PD-L1: 94 ± 2%, 83 ± 5%, 62 ± 8%, respectively, $p < 0.001$). BTLA was expressed by 93–100% of B-cells, and mean fluorescence intensity was significantly higher in plasma-cells (334 ± 146 versus 127 ± 12 in naïve B-cells, $p = 0.048$). BCR activation enhanced the expression all three receptors in normal B-cells; addition of CpG-ODN (TLR-9 ligand) further induced PD-1 and PD-L1-but not BTLA–expression, whereas addition of the cytokines IL-4, IL-10, or IL-21 reduced PD-1 and BTLA levels. In vitro crosslinking of BTLA resulted in reduction of BCR-induced phosphorylation of ERK, CD80/CD86 and BAFF-receptor expression, as well as in inhibition of cell proliferation (divided cells: 5.3 ± 0.4% versus 17.7 ± 0.1% in anti-IgM-stimulated cells). In comparative analysis, SLE patients exhibited significantly higher PD-1 expression on plasma-cells compared to healthy donors (65 ± 5% versus 36 ± 7%, $p = 0.002$), whereas there was no difference in PD-L1 or BTLA. Preliminary studies suggest distinct roles for PD-1 and BTLA in regulation of activation and maturation of B-cells in healthy controls and in the context of lupus.

Conclusions The coinhibitory receptors PD-1, PD-L1 and BTLA demonstrate differential expression among B-cell subsets and they are induced upon stimulation with important implications for the regulation of B-cell activation, proliferation and differentiation. Aberrancies in the expression and function of coinhibitory receptors in SLE plasma B-cells could contribute to enhanced autoantibody-forming capacity and disease pathogenesis.

A5.27 Ro52 EXPRESSION IS A PROGNOSTIC FACTOR FOR SURVIVAL IN B CELL LYMPHOMA

doi:10.1136/annrheumdis-2013-203219.27

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Background and Objectives The risk for lymphoma is increased in many rheumatic conditions. Diffuse Large B-cell Lymphoma (DLBCL) is a common, yet heterogeneous lymphoma subtype and good prognostic biomarkers are warranted. We have previously shown that the rheumatic autoantigen Ro52 is expressed predominantly in leukocytes and that over expression affects lymphocyte proliferation. Further, the Ro52 (*Trim21*) gene maps to a tumour suppressor locus. We therefore hypothesised that Ro52 expression might be used as a prognostic marker for lymphoma.

Materials and Methods Proliferation of in vitro stimulated lymphocytes was determined by thymidine incorporation and cell cycle analysis and compared to Ro52 mRNA levels. Three cohorts of patients with DLBCL were investigated for Ro52 expression by immunohistochemistry using monoclonal Ro52 antibodies. The first cohort consisted of patients with rheumatic disease treated with cytostatic drugs only (n = 43). The two others were non-rheumatic patients treated with anthracycline-based (CHOP) therapy alone (n = 74), and with the addition of Rituximab (n = 196).

Results A robust inverse correlation between Ro52 expression levels and cellular proliferation was observed ($r^2 = 0.50$, $p < 0.0001$). Confirming this biological phenomenon, low expression of Ro52 in lymphoma tissue was significantly correlated to overall survival ($p < 0.0001$), as well as progression-free survival ($p < 0.0001$) in patients with DLBCL treated with CHOP therapy alone. This was further confirmed in the Rituximab-CHOP treated cohort, with a significantly lower overall survival ($p < 0.0001$) and progression-free survival ($p = 0.0005$). The association was independent of Ann Arbor classification and DLBCL subtype, emphasising that Ro52 expression is a parameter of added value in lymphoma investigations to understand the patient prognosis.

Conclusions Our data demonstrate that low Ro52 expression is associated with more aggressive lymphomas. Further, our data functionally link low Ro52 expression with increased cellular proliferation. Ro52 may thus constitute a novel biologically relevant biomarker predicting patient survival in lymphoma.

A5.28 SEMAPHORIN3A IS A POTENT B CELL REGULATORY MOLECULE IN SLE

doi:10.1136/annrheumdis-2013-203219.28

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Background/Purpose Semaphorin3A (sem3A) is an important regulatory molecule, previously reported to play a role in the pathogenesis of rheumatoid arthritis and later by us in lupus glomerulonephritis. In addition, sema3A was shown to be a marker of T regulatory cells. A subpopulation of B cells, namely B regulatory cells (Bregs), was recently identified by us as $CD19+CD25^{high}IL\text{-}10^{high}TGF\text{-}\beta^{high}$, and demonstrated to be of high sema3A expression.

We therefore asked whether serum levels of sema3A is altered in SLE patients, whether sema3A expression on B regs of these patients is different, and finally, whether the addition of soluble sema3A to