Poster Presentations

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Innate immunity in rheumatic diseases

FRI0001

NEUTROPHILS IN GRANULOMATOSIS WITH POLYANGIITIS DISPLAY FEATURES OF PYROPTOSIS

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Background: Granulomatosis with polyangiitis (GPA) is characterized by extravascular necrotizing granulomatous inflammation and systemic ANCA – associated (AAV) vasculitis with neutrophils as a key player in the pathogenesis (1). We and others have shown that neutrophil-related cell death mechanisms contribute to chronic inflammatory processes in AAV (2, 3). Recently, another form of inflammatory cell death primarily described in monocytes called pyroptosis was also discovered in neutrophils (4). A cardinal feature of pyroptosis is the activation of the NLRP3 inflammasome, a sensor of different pathogen- and damage-associated molecular patterns (PAMP, DAMP), following caspase-1-mediated processing and secretion of IL-1beta (5).

Objectives: The aim of this study was to investigate, if neutrophils from GPA patients express pyroptosis-related components NLRP3, active caspase 1 and cleaved IL-1beta.

Methods: Polymorphonuclear leukocytes (PMN) were isolated from peripheral blood of GPA patients and healthy controls (HC) (n = 10 each). Expression of NLRP3, inactive/active caspase 1 and active IL-1beta was determined by western blot. In addition, peripheral blood mononuclear cells (PBMC) were isolated from GPA and HC. mRNA expression of *nlrp3* and *il1b* was determined by qPCR. To exclude false-positive results by contamination with monocytes we performed flow cytometry analysis of whole blood samples with markers CD3, CD14, CD15, CD66b and NLRP3.

Results: PMN from GPA patients showed markedly increased expression of NLRP3, active caspase 1 and active IL-1beta compared to HC. In contrast, there was no difference between GPA and HC on the mRNA level of neither *nlrp3* nor *il1b* in PBMC. In addition, we confirmed by flow cytometry increased expression of NLRP3 in PMN from GPA, but not in monocytes.

Conclusion: Here we provide evidence, that neutrophils from GPA undergo pyroptosis, demonstrated by increased NLRP3, active caspase 1 expression as well as IL-1beta processing. Neutrophils are present in high numbers at the site of granulomatous lesions of inflamed tissue in GPA and IL-1beta is increased in GPA sera (2). Therefore, neutrophils represent a potential source of IL-1beta in GPA. Given the fact that GPA-associated features such as massive release of necrosis-related DAMP or microbial agents such as *Staphylococcus aureus* (6) can activate the NLRP3-inflammasome, we identified here a potential relevant mechanism of neutrophils contributing to chronic inflammation of GPA.

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FRI0002

S100A11 (CALGIZZARIN) IS RELEASED DURING NEUTROPHIL EXTRACELLULAR TRAPS FORMATION AND STIMULATES RELEASE OF IL-6 AND TNF IN RHEUMATOID ARTHRITIS

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Background: S100A11 protein, a member of S100 family, has been associated with several autoimmune inflammatory conditions such as rheumatoid arthritis (RA). Although the pathogenesis of autoimmune diseases is not fully understood, the formation of neutrophil extracellular traps (NETs) seems to play a certain role. Recent data indicate that S100A8/A9 is released via NETosis and can further augment inflammatory responses.

Objectives: The aim of our study was to examine the association of S100A11 with NETs in RA.

Methods: To assess the expression of S100A11 by neutrophils of RA synovial tissue (n=8), immunofluorescence staining of S100A11 and myeloperoxidase (MPO) was performed. The levels of S100A11 and MPO in RA synovial fluid (n=23) were measured by ELISAs (RayBiotech and Abcam), and the activity of peptidyl arginine deiminases (PADs) was measured by an in-house immunoassay. NETosis was induced by adding phorbol 12-myristate 13-acetate (PMA) to neutrophils from RA patients (n=7). Release of NETs was visualised by immunocytochemistry (n=7) and the presence of S100A11 in supernatants was analysed by ELISA (RayBiotech). Neutrophils purified from healthy donors (n=5) were stimulated by S100A11 and the release of cytokines TNF and IL-6 was measured by ELISA (RayBiotech).

Results: S100A11 was expressed by synovial tissue neutrophils of the RA patients (n=8). The levels of S100A11 in the synovial fluid of RA patients (n=23) correlated with the levels of a NETosis marker MPO (r=0.562, p=0.005) and with PADs activity (r=0.690, p<0.001), which affects NETs immunogenicity. Neutrophils treated with LPS (n=7) did not up-regulate the secretion of S100A11 compared to unstimulated controls (0.23±0.05 vs. 0.29±0.07 ng/ml; p=ns). However, the release of S100A11 was markedly up-regulated in PMA-stimulated neutrophils undergoing NETosis compared to untreated controls (1.16±0.17 vs. 0.29±0.07 ng/ml; p<0.001). Moreover, diphenyleneiodonium treatment abolished PMA-induced S100A11 secretion. By immunofluorescence staining (n=8) we demonstrated that neutrophils activated by PMA release NETs containing S100A11 protein. In addition, extracellular S100A11 augmented the inflammatory response of neutrophils from healthy donors (n=5) via IL-6 and TNF in comparison with unstimulated cells (0.39±0.11 vs. 0.05±0.01 pg/ml; p<0.05 and 0.31±0.06 vs. 0.09±0.03 pg/ml; p<0.05).

Conclusion: Here we show for the first time that release of S100A11 by neutrophils is dependent on NETosis. Moreover, extracellular S100A11 augments the inflammatory response by inducing TNF and IL-6 secretion in neutrophils.

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FRI0003

ELEVATED HISTONE H4 IN NEUTROPHIL EXTRACELLULAR TRAPS PROMOTES MACROPHAGE ACTIVATION IN BEHÇET'S DISEASE

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Background: Neutrophil-released neutrophil extracellular traps (NETs) are upregulated and promote autoinflammation and thrombosis in Behçet's disease (BD), a multi-system inflammatory disease with unknown etiology^{1,2}. However, whether NETs promote macrophage activation in BD remains unclear.

Objectives: To investigate the potential role of NETs in promoting aberrant macrophage activation in BD.

Methods: We quantified NETs by measuring dsDNA using ELISA and immunofluorescence. Macrophages were stimulated with BD- and healthy controls (HC)-derived NETs, and IL-8 and TNF- α production were measured by ELISA. NETs-stimulated macrophages were incubated with naive CD4⁺ T cells, and Th1 cell differentiation was examined on day 7 by flow cytometry. Histones H1, H2A, H2B, H3, H4, S100A8 and neutrophil elastase in NETs were analyzed by western blot. Macrophages were stimulated with anti-Histone 4 antibody-treated NETs, and IL-8 production was measured by ELISA.

Results: Circulating NETs (2336 $\pm$ 534 ng/ml vs. 1472 $\pm$ 549 ng/ml, $P=0.0008$) and neutrophil-derived NETs (909.2 $\pm$ 485.2 ng/ml vs. 582.4 $\pm$ 199.2 ng/ml, $P=0.0108$) were significantly higher in BD patients compared with those in HC. BD NETs stimulated macrophages to produce a higher level of IL-8 (17 $\pm$ 4 ng/ml vs. 13 $\pm$ 4 ng/ml, $P=0.0474$) and TNF- α (166 $\pm$ 61 pg/ml vs. 102 $\pm$ 48 pg/ml, $P=0.0132$) than HC NETs. Moreover, BD NETs promoted macrophages to facilitate Th1 differentiation than HC NETs (33 $\pm$ 10% vs. 24 $\pm$ 7%, $P=0.0398$). Western blot analysis revealed more Histone H4 (289076 (144365, 544038) IOD values vs. 42121 (6958, 129625) IOD values, $P=0.0286$), but not Histones H1, H2A, H2B, H3, S100A8 or neutrophil elastase in BD NETs compared to HC NETs. Importantly, neutralizing Histone H4 abrogated the BD NETs-stimulated IL-8 overproduction by macrophages (9.99 $\pm$ 2.07 ng/ml vs. 13.95 $\pm$ 2.91 ng/ml, $P=0.021$).

Conclusion: BD NETs promoted macrophages activation, which might be mediated by a higher level of Histone H4.

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FRI0004

SURFACE AMP DEAMINASE 2 AS A NOVEL REGULATOR MODIFYING THE EXTRACELLULAR ATP-ADENOSINE BALANCE THAT IS DIFFERENTIALLY EXPRESSED IN PATIENTS WITH RHEUMATOID ARTHRITIS

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Background: Adenosine and its nucleotides represent crucial immunomodulators in the extracellular environment. ATP and ADP are released from stressed cells in states of inflammation, whereas adenosine serves as a key anti-inflammatory mediator¹. The ectonucleotidases CD39 and CD73 are responsible for the sequential catabolism of ATP to adenosine via AMP, thereby promoting an anti-inflammatory milieu induced by the "adenosine halo". Great importance has been attributed to these enzymes in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA) and as targets in cancer therapy^{2,3}. AMPD2 mediates AMP deamination to IMP, thus constituting an ambiguous mediator both enhancing the degradation of inflammatory ATP and reducing the formation of protective adenosine. Here, we postulate that this pathway is also present on the cell surface of immune cells and modified under inflammatory conditions.

Objectives: Therefore, we analysed surface AMPD2 expression and its modulation on distinct cell lines and primary immune cells.

Methods: Firstly, AMPD2 surface expression was verified by immunoprecipitation from membrane fractions isolated from cell lines (HEK293 and HMEC1) and CD14⁺ monocytes analysed by western blot and mass spectrometry. In addition, surface biotinylation of the aforementioned cells was performed. Also, AMPD2 surface expression was evaluated by flow cytometry, analysing both cell lines (HEK293, HMEC1, THP1, and Jurkat) and primary human immune cells from healthy donors and patients with RA.

Secondly, co-expression of surface AMPD2, CD39 and CD73 on PBMCs was analysed by flow cytometry directly after isolation as well as after a 24h culture period. Moreover, surface expression was assessed after immunostimulation and Golgi transport inhibition.

Results: AMPD2 surface expression was confirmed by western blot and mass spectrometry of (i) precipitated AMPD2 from membrane fractions and (ii) biotinylated surface molecules in HEK293 and HMEC1 as well as CD14⁺ monocytes. Surface expression was reduced after AMPD2 knockdown in HEK293. Flow cytometric analysis further verified AMPD2 surface expression and revealed a significant decrease after Golgi transport inhibition ($p<0.01$). TLR

Figure 1. Surface AMPD2 as a novel regulator of the extracellular ATP-adenosine balance.

stimulation strongly enhanced the surface expression of AMPD2 and CD39 on monocytes ($p<0.05$), whereas dexamethasone at high therapeutic doses inversely affected AMPD2 surface expression on lymphocytes and monocytes ($p<0.01$). Analysis of AMPD2 surface expression on PBMCs from RA patients revealed higher expression levels compared to sex- and age-matched healthy controls ($p<0.05$).

Conclusion: We demonstrate AMPD2 surface expression on immune cells for the first time. Hence, we reveal a novel regulator of the extracellular ATP-adenosine balance that is differentially expressed in RA patients compared to healthy controls. The extracellular conversion of AMP into IMP may constitute a shunt-like mechanism adding to the CD39-CD73 system controlling immunomodulation.

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FRI0005

DIVERSITY OF ANTI-CITRULLINATED PROTEIN ANTIBODY COMPOSITIONS INFLUENCE SYNOVIAL FIBROBLAST REACTIVITY

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Background: Anti-citrullinated protein antibodies (ACPAs) play an important role in rheumatoid arthritis (RA) pathogenesis. We hypothesized that the effect of these antibodies is mediated by their binding to synovial fibroblasts and inducing an increased mobility of fibroblasts¹.

Objectives: In our study, we analyzed and compared fibroblast modulation by ACPA pools obtained from different patients or by a set of monoclonal ACPAs with different fine specificity that were obtained from different tissue sites.

Methods: Synovial fibroblasts were isolated from RA patients synovial tissue biopsies. Individual polyclonal ACPA and control IgGs were purified from sera of four ACPA-positive RA patients by affinity purification on protein G and CCP-2 columns. Monoclonal antibodies were derived from memory B cell isolated from blood2, synovial fluid or bronchoalveolar lavage of RA patients. Whole antibodies and F(ab')₂ fragments were tested in fibroblast migration using IncuCyte live-cell analysis. Blocking experiments were performed with soluble citrullinated proteins in SF migration. Cross-reactivity of the antibodies to citrullinated and acetylated epitopes was tested using PAD inhibitors (CI-amidine and GSK199),