PROF. PAUL PROOST (Orcid ID : 0000-0002-0133-5545)

Article type : Full Length

Synovial fluid neutrophils from patients with juvenile idiopathic arthritis display a hyperactivated phenotype

Running head: Neutrophils in juvenile idiopathic arthritis

Mieke Metzemaekers, MSc[1], Bert Malengier-Devlies, MSc[2], Karen Yu, MSc[1], Sofie Vandendriessche, MSc[1], Jonas Yserbyt, MD, PhD[3], Patrick Matthys, MSc, PhD[2], Lien De Somer, MD, PhD[2,4,5,6], Carine Wouters, MD, PhD[2,4,5,6], Paul Proost, MSc, PhD[1,6*]

[1]Laboratory of Molecular Immunology, Rega Institute, KU Leuven, Leuven, Belgium

[2]Laboratory of Immunobiology, Rega Institute, KU Leuven, Leuven, Belgium

[3]Department of Respiratory Diseases, University Hospitals Leuven, Leuven, Belgium.

[4]Division of Pediatric Rheumatology, University Hospitals Leuven, Leuven, Belgium

[5]European Reference Network for Rare Immunodeficiency, Autoinflammatory and Autoimmune Diseases (RITA) at University Hospital Leuven, Leuven, Belgium

[6]equally contributing senior authors

*Correspondence to: Prof. Paul Proost, Lab. Molecular Immunology, Rega Institute, KU Leuven, Herestraat 49, Box 1042, Leuven, Belgium; email – paul.proost@kuleuven.be; phone – +32 16 32 24 71

Grant support:

This work was supported by the Research Foundation - Flanders (FWO-Vlaanderen) (G.0808.18N), a "C1" grant (C16/17/010) from KU Leuven, the Rega Foundation and received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 779295. M.M. obtained a PhD fellowship supported by the L'Oréal – UNESCO for Women in Science initiative and the FWO-Vlaanderen. S.V. is recipient of a PhD SB fellowship of FWO-Vlaanderen.

Financial Interests:

J.W. obtained unrestricted grants to KU Leuven from Novartis, Roche, GSK immuno-inflammation and Pfizer.

Word count:

4163

Abstract

Objectives. Juvenile idiopathic arthritis (JIA) is the most common rheumatic disease in childhood. The predominant subtypes, i.e. oligoarticular (oligo) and polyarticular (poly) JIA, are traditionally considered to be autoimmune diseases with a central role for T cells and autoantibodies. Mounting evidence suggests an important role for neutrophils in JIA pathogenesis. Here, we investigated the phenotypical features of neutrophils present in the blood and inflamed joints of patients.

Methods. Synovial fluids and parallel blood samples from JIA patients and blood samples from healthy children were collected. Synovial fluid-treated healthy donor neutrophils and pleural neutrophils from patients with pleural effusion were investigated as controls for synovial fluid exposure and extravasation. Multicolor flow cytometry panels allowed for in-depth phenotypical analysis of neutrophils, focusing on the expression of adhesion molecules, activation and maturation markers and chemoattractant receptors. Multiplex technology was exploited to quantify cytokines in plasma and synovial fluids.

Results. Synovial fluid neutrophils displayed an activated, hypersegmented phenotype with decreased CD62L expression, upregulation of adhesion molecules CD66b, CD11b and CD15 and downregulation of CXCR1/2. An elevated percentage of CXCR4 positive neutrophils was detected in synovial fluids from patients. Pleural neutrophils showed less pronounced maturation differences. Strikingly, significant percentages of synovial fluid neutrophils showed a profound upregulation of atypical neutrophil markers, including CXCR3, ICAM-1 and HLA-DR.

Conclusion. Our data show that neutrophils in inflamed joints of JIA patients have an activated phenotype. This detailed molecular analysis supports the notion that a complex intertwining between these innate immune cells and adaptive immune events drives JIA.

Key words: juvenile idiopathic arthritis, neutrophil, cytokines, chemokines, adhesion molecule

Introduction

Juvenile idiopathic arthritis (JIA) is the most common rheumatic disease in childhood and an important cause of disability if patients are not treated appropriately (1). By definition, JIA clinically presents with peripheral joint inflammation of unknown origin, persisting for at least six weeks and starting before 16 years of age. The disease is categorized into distinct subclasses, with oligoarticular (oligo) and polyarticular (poly) JIA being most prevalent. Apart from the number of inflamed joints during the first six months of disease, some mechanistic and immunological overlap exists between these two JIA subtypes. Consistently, there is growing consensus that oligoJIA and in particular, rheumatoid factor negative polyJIA may represent a continuum of a single disease entity rather than distinct diseases. Oligo- and polyJIA are traditionally considered to be multifactorial autoimmune diseases that presumably start when a rather innocuous environmental trigger evokes an autoreactive insult in genetically predisposed individuals (2,3). The strong association with HLA variants and profound accumulation of Th1 cells in affected joints underline the pivotal role of the adaptive immunity in these JIA subtypes (1,4). Nevertheless, the full spectrum of immune-pathological manifestations observed in patients most likely involves both innate and adaptive immunity (4,5). Previous research efforts showed that peripheral blood neutrophils from JIA patients display transcriptional abnormalities and remain in an activated state even during remission, suggesting a role for neutrophils in JIA pathophysiology (6–8). This hypothesis is further supported by the presence of S100A12 in serum and synovial fluids of patients (9). Neutrophils are armed with oxidative and non-oxidative defense mechanisms, including the capacity to release reactive oxygen species and the ability to engulf and destroy foreign material (phagocytosis) (10). These innate leukocytes were initially considered simple and mere servile phagocytes, acting as non-specific pathogen exterminators only. With the appearance of sophisticated technologies, a more refined model of neutrophil function has evolved. Mounting evidence suggests that neutrophils display phenotypical and functional heterogeneity and may contribute to the initiation, modulation and resolution of inflammation, either directly or indirectly via instructing innate as well as adaptive immune cells (11–18). Moreover, strongly activated neutrophils may eventually acquire the capacity to present antigens (19,20). The precise phenotypical features of neutrophils from JIA patients, in particular those present in the synovial fluid of inflamed joints, are currently unknown. We therefore aimed to characterize extensively the circulating and synovial neutrophils from oligo- and polyJIA patients.

Patients & Methods

Patients

Twenty-three JIA patients with a median age (range) of 11 (4-17) years and female/male ratio of 15/8 were recruited at the University Hospital Leuven. Synovial fluid was collected only if joint aspiration was required for treatment. Blood and synovial fluid samples were collected in BD Vacutainer tubes treated with ethylenediaminetetraacetic acid (EDTA) (BD Biosciences) and processed within 30 min of withdrawal. Pleural fluids were collected from five patients that required local aspiration with a median age (range) of 68 (41-81) and female/male ratio of 2/3 and were studied as a control for neutrophil extravasation. In addition, blood samples were collected from twelve healthy children [median age (range) of 10 (4-18) years and female/male ratio of 7/5] and eighteen adult volunteers [median age (range) of 28 (24-64) years and female/male ratio of 12/6]. Informed consent was obtained according to the ethical guidelines of the Declaration of Helsinki. Parents or legal guardians signed the informed consent on behalf of under-aged patients. The Ethics Committee of the University Hospital Leuven approved this study (S59874). Please refer to Suppl. Table S1 for detailed characteristics of patients. Due to the limited volumes of samples available from pediatric individuals, we were unable to use all samples for each set of experiments and thus the numbers of patients and controls analyzed differ for some experiments.

Cells and flow cytometry

The EasySep™ Direct Human Neutrophil Isolation Kit (Stemcell Technologies) was used for removal of erythrocytes from blood samples. Cells were treated with FcR block (Miltenyi Biotec) and Fixable Viability Stain 620 (BD Biosciences) or Zombie Aqua 516 (Biolegend), followed by extracellular staining. Antibodies used in this study are listed in Suppl. Table S2 and were titrated in-house. For intracellular staining of TLR9, cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), followed by staining with anti-human TLR9. Results were analyzed using a BD LSRFortessa™ X-20 (BD Biosciences) equipped with five lasers. For downstream analysis with FlowJo software, neutrophils were gated as $CD16^{+}CD66b^{+}$ cells within the population of living cells that occurred as singlets only.

Cytokine measurements

Plasma and synovial fluid concentrations of interleukin IL-6, IL-10, IL-12/IL-23 p40 subunit, IL-17A, IL-1 receptor antagonist (IL-1RA), tumor necrosis factor interferon (IFN-)γ, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF) and the chemokine CXCL8 were determined using customized Meso Scale Discovery (MSD) multiplex technology. Detection limits of cytokine assays are shown in Suppl. Table S3.

*E*x vivo stimulation of neutrophils

Freshly isolated peripheral blood neutrophils from adult healthy donors were exposed to cell-free plasma from the original donor or cell-free JIA synovial fluid, with or without 2 µg/ml anti-TNF (ethanercept; Pfizer), 2 µg/ml (anakinra, Sobi) and/or 2 (tocilizumab, Roche). Alternatively, cells were diluted in [Roswell Park Memorial Institute 1640 (RPMI1640) medium (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco) and 5 ng/ml GM-CSF (PeproTech)], in the presence or absence of 10 ng/ml 10 ng/ml 10 ng/ml IL-6 (Peprotech), with or without 2 µg/ml of the corresponding cytokine inhibitor. Cells were seeded in a 48-well plate at a final concentration of 2 x 10^6 cells/ml (total volume of 500 µl per well) and placed at 37°C with 5% CO_2 for 3 to 24 hours. Subsequently, cells were used for extensive phenotypical characterization by flow cytometry. Information on neutrophil survival under different culturing conditions is included in Suppl. Fig. S1 and S2A.

tatistics

Kruskal-Wallis with Dunn's multiple comparisons tests were performed to detect significant differences between the study groups. A p-value of 0.05 or less was considered significant.

ts

levated cytokine levels in synovial fluids from JIA patients

To explore the inflammatory environment, cytokine measurements were performed in synovial fluid samples from the affected joints and circulation of JIA patients. Circulating cytokine values from healthy children were used for comparative purposes. Synovial fluids contained significantly elevated concentrations of the pro-inflammatory cytokines IL-6, IL-12/IL-23p40, and as compared to plasma samples from either patients or controls (Table 1). In addition, some JIA patients displayed a tendency towards enhanced synovial levels of

IL-17A but no significant differences were detected. Concentrations of the anti-inflammatory cytokines IL-10 and IL-1RA were significantly increased in synovial fluids as compared to plasma concentrations from patients or healthy children. Interestingly, the most powerful neutrophil-attracting chemokine in humans, i.e. CXCL8, was abundantly present in synovial fluids from JIA patients but not in plasma samples from patients and controls. Quantification of M-CSF and GM-CSF revealed that the concentration of these myelopoietic cytokines was significantly higher in synovial fluids as compared to plasma samples from patients or controls. No significant alterations in G-CSF concentrations were detected.

Synovial fluid neutrophils from JIA patients display a hypersegmented phenotype

Neutrophil maturation can be assessed based on the intensity of CD16 receptor III) and CD62L (L-selectin) expression (21,22). Three distinct neutrophil subsets were described, i.e. $CD16^{med}CD62L^{high}$ immature neutrophils with a banded nucleus, $CD16^{high}CD62L^{high}$ mature neutrophils containing three or accidentally four nuclear lobes and $CD62L^{low}$ hypersegmented cells with on average a larger number of nuclear lobes. As expected, the majority of circulating neutrophils isolated from the peripheral blood of patients and healthy donors were $CD16^{high}CD62L^{high}$ mature cells (Fig. 1A; Suppl. Fig. S3A). In contrast, most neutrophils in synovial fluids displayed a hypersegmented character as evidenced by a significantly enhanced percentage of $CD62L^{low}$ cells (Fig. 1B; Suppl. Fig S3A). Noteworthy, patients with polyarticular disease tended to contain an increased percentage of $CD62L^{low}$ cells in the circulation as well (Fig. 1B). An increased percentage of $CD62L^{low}$ neutrophils was also detected in pleural fluids from patients with pleural effusion (Fig. 1B). However, in contrast to synovial neutrophils, the vast majority of pleural neutrophils retained a $CD16^{high}CD62L^{high}$ phenotype (Fig. 1A). $CD16^{med}CD62L^{high}$ immature neutrophils represented only a minor percentage of the total neutrophil population in blood, synovial and pleural fluids (Fig. 1C; Suppl. Fig. S3A). Analysis of cytospin preparations confirmed the presence of hypersegmented neutrophils and the absence of immature cells in synovial fluids (Suppl. Fig. S3B). Synovial fluids and, to a lesser extent, pleural fluids but not blood samples from either patients or controls contained a significant population of CXCR4-expressing $CD11b^{high}CD62L^{low}$ neutrophils (Fig. 1D). This phenotype has been associated with neutrophil aging (23). The observed shift towards the CD62Llow, CXCR4 positive phenotype could be mimicked by exposing healthy donor neutrophils to either plasma from the corresponding donor or JIA synovial fluid during 24 hours, and was not neutralized by inhibitors

of and/or IL-6 (Fig. 1E-F). Ex vivo stimulation of healthy neutrophils with synovi fluids for 3 or 6 hours did not induce a similar phenotypical shift (Suppl. Fig. S2B-C). Cultured neutrophils progressively reduced their CD16 expression to levels detected on immature cells as escribed previously (Suppl. Fig. S3C) (22).

ltered expression of adhesion and MHC class II molecules on synovial fluid neutrophils from JIA patients

CD66b (carcinoembryonic antigen-related cell adhesion molecule 8) was significantly pregulated on synovial fluid neutrophils (Fig. 2A). CD62L shedding and upregulation of CD66b are hallmark features of neutrophil activation. Pleural neutrophils from patients with pleural effusion also displayed a tendency towards increased expression of CD66b (Fig. 2A). No pregulation of CD66b was detected if healthy donor neutrophils were cultured in plasma from the me donor or JIA synovial fluid during 24 hours (Suppl. Fig. S2D; Suppl. Fig. S4A). The fact that CD66b was upregulated on synovial and pleural neutrophils, but not on synovial fluid-treated healthy donor neutrophils, may implicate that the observed phenotypical alteration is related to extravasation (24). In addition to the activated phenotype, a notable percentage of synovial fluid neutrophils expressed the MHC class II surface receptor HLA-DR, indicating that these cells acquired the capacity to behave as professional antigen presenting cells (Fig. 2B). Pleural neutrophils also displayed upregulation of HLA-DR but data did not reach statistical significance (Fig. 2B). No significant upregulation of HLA-DR expression was observed on healthy donor neutrophils 24 hours after subcultivation in plasma or patient synovial fluids, suggesting that nor aging neither exposure to a cell-free inflamed environment alone is sufficient to induce pregulation of HLA-DR expression (Suppl. Fig. S2E; Suppl. Fig. S4B).

The presence of surface adhesion molecules on neutrophils is essential for their interaction with endothelial cells and subsequent extravasation into inflamed tissues. Quiescent neutrophils express CD11b (integrin $_M$) and CD15 (sialyl Lewis X), that can be upregulated upon activation by means of degranulation (25). As expected, CD11b and CD15 were present abundantly on neutrophils in blood samples from patients and controls (Fig. 2C-D). Compared to circulating patient neutrophils, the expression levels of the two major adhesion molecules on synovial neutrophils were significantly increased (Fig. 2C-D). In contrast, no significant alterations of CD11b and CD15 expression were detected on pleural neutrophils (Fig. 2C-D). Healthy donor neutrophils stimulated with synovial fluids from JIA patients for 24 hours were featured by

significantly lower surface levels of CD11b and CD15 (Suppl. Fig. S4C-D). However, a tendency towards enhanced CD11b and CD15 expression was detected if cells were treated with synovial fluid for 3 hours, but data did not reach statistical significance (Suppl. Fig. S2F-G).

The integrin subunit CD49d – that may play a role in neutrophil recruitment during inflammation (26) – was absent on most circulating neutrophils and a non-significantly enhanced percentage of CD49d-expressing neutrophils was present in synovial and pleural fluids (Suppl. Fig. S4E, left panel). Noteworthy, incubation of healthy donor neutrophils with synovial fluids from JIA patients – but not with plasma from the original donor – for 24 hours provoked a significant upregulation of CD49d, suggesting that upregulation of CD49d occurs in response to inflammation (Suppl. Fig. S2H; Suppl. Fig. S4E, right panel). The increased CD49d expression was reduced with anti-TNF- and disappeared upon treatment with anti-IL-1 and/or anti-IL-6 (Suppl. Fig. S4E, right panel). As expected, circulating neutrophils from patients and controls did not express intercellular adhesion molecule-1 (ICAM-1) (Fig. 2E, left panel). However, a significant portion of synovial fluid neutrophils was ICAM-1 positive (Fig. 2E, left panel). It was demonstrated previously that upregulation of ICAM-1 boosts neutrophil effector functions (27). A tendency for an increased percentage of ICAM-1 expressing neutrophils was found in pleural fluids. Strikingly, incubation of healthy donor neutrophils with patient synovial fluids, but not with plasma, during 24 hours resulted in profound upregulation of ICAM-1, independently of the presence of and/or IL-6 inhibitors (Fig. 2E, right panel; Suppl. Fig. S2I).

Altered chemoattractant and Toll-like receptor expression profile on synovial fluid **neutrophil**s from JIA patients

Analysis of CXCR1 and CXCR2 expression, both high-affinity receptors for CXCL8, revealed a significantly reduced expression level of CXCR1 and a trend towards downregulation of CXCR2 on synovial neutrophils from JIA patients but not on pleural neutrophils from patients with pleural effusion (Fig. 3A-B, left panels). A trend towards decreased expression of both receptors was observed if peripheral blood neutrophils from JIA patients were compared to control cells (Fig. 3A-B, left panels). Analysis of healthy donor neutrophils following ex vivo culturing in plasma from the same donor revealed spontaneous downregulation of CXCR1 and CXCR2 after 24 hours, suggesting that the observed downregulation of the two receptors on synovial neutrophils is related to aging (Fig. 3A-B, right panel; Suppl. Fig. S2J-K). A minor but significant percentage of synovial fluid neutrophils stained positive for CXCR3 (Fig. 3C, left panel). CXCR3

is not typically expressed by neutrophils but may be upregulated during severe inflammation. A trend towards increased levels of CXCR3 positive neutrophils was observed in pleural fluids, but no significant differences were detected (Fig. 3C, left panel). Interestingly, upregulation of CXCR3 on healthy donor neutrophils was found if cells were exposed to synovial fluid from JIA patients during 24 hours (Fig. 3C, right panel; Suppl. Fig. S2L).

Compared to blood neutrophils from healthy children, complement receptor C5aR was significantly less abundant on synovial fluid neutrophils from JIA patients (Fig. 4A, left panel). Blood neutrophils from JIA patients displayed a trend towards downregulation of C5aR. Since C5aR is internalized rapidly upon exposure to high ligand concentrations; downregulation of the receptor at the protein level may indicate neutrophil activation. No alterations of C5aR expression were observed if pleural neutrophils were assessed. Synovial fluid neutrophils from JIA patients exhibited higher levels of the formyl peptide receptor FPR1 as compared to blood neutrophils from healthy children (Fig. 4B, left panel). Moreover, expression of FPR1 on pleural neutrophils was significantly increased as compared to blood neutrophils from healthy volunteers (Fig. 4B, left panel). Upregulation of FPR1, but not downregulation of C5aR, was mimicked if healthy donor neutrophils were cultured in synovial fluids from JIA patients during 24 hours (Fig. 4A-B, right panels; Suppl. Fig. S2M-N). The leukotriene B_4 receptor BLTR1 was significantly upregulated on synovial fluid neutrophils – but not on synovial fluid-treated healthy donor cells – as compared to blood neutrophils from JIA patients, (Fig. 4C; Suppl. Fig. S2O). Pleural neutrophils also displayed significantly elevated BLTR1 expression (Fig. 4C, left panel). Synovial fluid neutrophils showed a moderate but significant upregulation of TLR2 (Suppl. Fig. S5A). A trend towards increased TLR4 and TLR6 expression, was observed on synovial fluid and blood neutrophils from JIA patients, but no significant differences were detected (Suppl. Fig. S5B-C). The expression level of TLR9 was similar in all study groups (Suppl. Fig. S5D).

Correlations between phenotypical alterations of synovial neutrophils and local cytokine concentrations

Correlation studies were performed to evaluate potential associations between phenotypical characteristics of synovial neutrophils from JIA patients and the local cytokine concentrations. In addition, we exposed healthy donor neutrophils to three prototypical pro-inflammatory cytokines that are relevant therapeutic targets in the context of JIA and present at high concentrations in synovial fluids, i.e. IL-6 and (Table 1). Synovial levels of IL-6 ($p = 0.0015$) and II

1RA ($p = 0.0014$) and, to a lesser extent, ($p = 0.0031$) and CXCL8 ($p = 0.0040$), correlated positively with the abundance of $CD62L^{low}$ neutrophils in synovial fluids and tended to correlate with expression of CXCR4 (Fig. 5). Nevertheless, CD62L shedding and upregulation of CXCR4 so occurred spontaneously if healthy donor cells were cultured in plasma from the same donor during 24 hours (Fig. 1E-F). Treatment with or IL-6 had no additional effects on CD62L shedding and upregulation of CXCR4 by healthy donor neutrophils as compared to medium alone (Suppl. Fig. S6A-B). Collectively, these results suggest that CD62L shedding and upregulation of CXCR4 expression presumably coincide with neutrophil aging in synovial fluids of JIA patients.

A significant correlation between increased CD66b expression on synovial fluid neutrophils from JIA patients and enhanced local concentrations of IL-6 ($p = 0.0001$), IL-10 ($p = 0.0001$), CXCL8 ($p = 0.0019$) and ($p = 0.0003$) was uncovered (Fig. 5). Nevertheless, exposure of healthy donor neutrophils to synovial fluids from JIA patients, containing high concentrations of these cytokines, did not provoke upregulation of CD66b on healthy donor neutrophils (Suppl. Fig. S4A). Likewise, stimulation of healthy donor cells with or IL-6 had no significant effect on the intensity of CD66b expression (Suppl. Fig. S6C). In contrast to cells treated with IL-1 or IL-6, cells tended to exhibit elevated levels of CD66 (0.0824) (Suppl. Fig. S6C). No effect of the three cytokines was observed on HLA-DR expression on neutrophils (Suppl. Fig. S6D). Moreover, no significant associations were found between expression of CD11b, CD15, CD49d, ICAM-1, CXCR1/2/3, BLTR1, FPR1, C5aR and HLA-DR on synovial fluid neutrophils from JIA patients and the presence of a particular cytokine (Fig. 5).

Evaluation of CD11b expression on healthy donor neutrophils 24 hours after stimulation with TN or IL-6 revealed that but not or IL-6, induc upregulation of CD11b (Suppl. Fig. S7A). As expected, the upregulation of CD1 was blocked upon administration of the inhibitor ethanercept. No significant changes in expression level of CD15 or CD49d and ICAM-1 positive cells were detected upon incubation with TN or IL-6, although a trend towards increased ICAM-1 expressing neutrophi was found if cells were treated with or (Suppl. Fig. S7B-D). All three cytokines failed to interfere with expression of chemoattractant receptors CXCR1, CXCR2 and FPR1 (Suppl. Fig. S8A-C). In addition, stimulation of healthy donor neutrophils with IL-6 had no significant effects on the percentage of CXCR3-expressing cells, although a trend towards increased numbers of CXCR3 positive neutrophils was observed upon treatment with

(Suppl. Fig. S8D). neutrophils showed a significant downreg
C5aR and a trend towards increased BLTR1 expression (Suppl. Fig. S8E-F). Both
mediated effects were blocked in the presence of ethanercept.

Discussion

Oligo- and polyJIA have long been assumed autoimmune diseases caused by dysregulation of the adaptive immune system, with a central role for autoreactive T cells belonging to the Th1 and Th17 lineages and autoantigens that may include aggrecan, fibrillin, matrix metalloproteinase (MMP)-3 and heat shock proteins (28–33). Nevertheless, the original T cell-centered hypothesis has been challenged since it does not cover nor completely explain the full spectrum of immune-pathological phenomena observed in patients. Moreover, successful therapeutic strategies are either non-specific (such as corticosteroids, methotrexate) or rather target downstream of the inflammatory cascade just one inflammatory cytokine blockers, IL-6 blockade) or T cell interaction (CTLA-4 Ig).

Former research efforts revealed that peripheral blood neutrophils from polyJIA patients are chronically activated cells featured by transcriptional abnormalities – including disruption of gene regulatory networks in clusters of and CXCL8-modulated genes – even during remission (6,7). Moreover, a correlation was demonstrated between S100A12 serum levels and disease activity (9). Interestingly, associations between effective medical treatment and extensive modulation of the neutrophil transcriptional profile were unveiled (8). These observations suggest an important role for neutrophils in JIA pathogenesis, spiking our interest to investigate the phenotypical characteristics of neutrophils present in the circulation and in inflamed joints from JIA patients.

Evidence for neutrophil activation at the protein level has only been published for patients with systemic-onset JIA, that is believed to be an autoinflammatory disease driven mainly by aberrant innate immune responses (34). The present study is the first to characterize neutrophils, present in blood and synovial fluids of oligo- and polyJIA patients, at the protein level. Our results indicate the existence of strongly activated neutrophils in synovial fluids from JIA patients, featured by CD62L shedding, upregulation of CD66b, CD11b and CD15, downregulation of CXCR1 and upregulation of alternative surface molecules including, but not limited to, HLA-DR and ICAM-1. In pleural fluids, neutrophil activation was less evident since no significant

alteration in CD66, CD11b, CD15 or CXCR1 expression was detected. Neutrophils were not or hardly activated in peripheral blood of JIA patients at the protein level. This corresponds to the absence of major signs of systemic inflammation at the moment of sampling (as evidenced by low CRP and ESR levels shown in Suppl. Table S1 and plasma cytokine concentrations depicted in Table 1).

Importantly, downregulation of CD62L and CXCR1/2 occurred spontaneously if healthy donor neutrophils were cultured for 24 hours in plasma from the corresponding donor, indicating that these phenotypical changes presumably coincide with neutrophil aging. Moreover, upregulation of CD49d, ICAM-1, FPR1 and CXCR3 was mimicked if healthy donor neutrophils were exposed to synovial fluids from JIA patients, suggesting that the local milieu with enhanced concentrations of pro-inflammatory mediators is at least partially responsible for the increased expression of these molecules on synovial neutrophils. Synovial fluid neutrophils from JIA patients, but not plasma- or synovial fluid-treated healthy donor cells, are featured by upregulation of CD66b, HLA-DR, CD11b, CD15 and BLTR1 and downregulation of C5aR. Pleural neutrophils from patients with pleural effusion also displayed a trend towards higher surface levels of HLA-DR expression and decreased expression of C5aR. Therefore, these phenotypical alterations may be related to neutrophil extravasation.

Analysis of nuclear segmentation properties revealed an increased percentage of hypersegmented neutrophils in the synovial fluids from JIA patients. The functional characteristics of this particular neutrophil subset are poorly defined. Neutrophils with increased numbers of nuclear lobes are believed to be capable of suppressing the proliferation of T cells via CD11b/CD18-dependent release of hydrogen peroxide and show a unique proteome profile (21, 35). Hypersegmentation was proposed to be associated with neutrophil aging. Nevertheless, it was speculated that banded cells may mature directly into hypersegmented neutrophils under particular pathophysiological circumstances (36). In addition, it was suggested that $CD62L^{low}$ hypersegmented neutrophils, present in the bone marrow, enter the circulation only during acute inflammation (21). Thus, it remains an open question whether all $CD62L^{low}$ hypersegmented neutrophils truly are aged cells.

Functionally, it was demonstrated that $CD62L^{low}$ aged neutrophils display an impairment in induction of shape change and chemotaxis, but enhanced surface expression of CD18 and increased hydrogen peroxide production (37). Evidence for the presence of aged neutrophils in

inflamed joints from JIA patients was also provided when the expression of chemokine receptors was investigated. Our results show a significant portion of CXCR4 positive neutrophils present in JIA synovial fluids. Upregulation of CXCR4 on synovial fluid neutrophils from patients with various forms of arthritis was previously reported and is a hallmark feature of aged neutrophils, allowing them to home back to the bone marrow following senescence (38). Although aged neutrophils show diminished pro-inflammatory activity in vitro, they presumably represent a population of superior mediators of inflammation in vivo (23,37,39,40).

Neutrophils may upregulate MHC class II molecules on their cellular surface, presumably in a CD11b-dependent fashion and in response to T cell-derived cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-3 (19,20,41,42). MHC class II-expressing neutrophils may actively present antigens to T cells, suggesting that they are potentially critical players acting at the intersection between innate and adaptive immunity (43–45). The existence of MHC class II-positive neutrophils in vivo has been evidenced in the context of rheumatoid arthritis (41). Here, we report the presence of HLA-DR-expressing neutrophils in inflamed joints from JIA patients. This subset of neutrophils may fulfill a key role in regulating the adaptive immune response, favoring the idea that JIA is driven by an intriguing interplay between innate and adaptive immune cells.

ICAM-1 is generally absent on neutrophils or expressed only at very low levels. Exposure to inflammatory signals including lipopolysaccharide (LPS) and evokes upregulation of ICAM-1 on neutrophils (27). Functionally, ICAM-1-expressing neutrophils excel in terms of ROS production, phagocytic capacity and ability to release neutrophil extracellular traps (27,46). Our observation that a significant portion of synovial fluid neutrophils from JIA patients are ICAM-1-positive cells suggests the presence of a subpopulation with altered pro-inflammatory activity.

In conclusion, the present study is the first to provide a comprehensive phenotypical profile of neutrophils present in the circulation and inflamed joints from oligo- and polyJIA patients. Our data indicate that synovial neutrophils have an activated phenotype, and let us speculate that neutrophils are important orchestrators of innate and adaptive immune events in JIA pathogenesis.

Table 1. Cytokine levels in plasma and synovial fluids from JIA patients.

Cytokine/chemokine	PedCo	JIA	JIA SF
IL-1β (pg (pg/ml)	< 1	< 1 (< 1 – 46.5)	7.7 (< 1 – 52.2)$^{***, \$\$\$\$}$
	n = 10	n = 16	n = 21
IL-6 (pg/ml)	0.7 (< 0.5 – 4.1)	1.4 (< 0.5 – 17.8)	1602 (53.8 – 7329)$^{****, \$\$\$\$}$
	n = 10	n = 16	n = 21
IL-12/IL-23P40 (pg/ml)	262.8 (172.3 – 483.1)	285.5 (134.1 – 570.2)	1387 (97.3 – 2156)$^{*, \$\$}$
	n = 5	n = 8	n = 13
IFN-γ (p (pg/ml)	18.2 (< 9 – 105.0)	10.8 (< 9 – 74.5)	137.1 (< 9 – 490.6)$^{*, \$\$\$\$}$
	n = 10	n = 16	n = 21
TNF-α (p (pg/ml)	2.8 (< 1 – 4.0)	2.1 (< 1 – 4.1)	10.5 (1.5 – 22.8)$^{*, \$\$\$\$}$
	n = 10	n = 16	n = 21
IL-17A (pg/ml)	< 4 (< 4 – 4.7)	< 4 (< 4 – 14.1)	9.3 (< 4 – 69.1)
	n = 5	n = 8	n = 13
IL-10 (pg/ml)	< 1 (< 1 – 3.9)	< 1 (< 1 – 1.1)	6.9 (< 1 – 22.3)$^{**, \$\$\$\$}$
	n = 10	n = 16	n = 21
IL-1RA (pg/ml)	278.0 (107.9 – 941.5)	160.7 (79.5 – 273.5)	3590. (398.5 – 11683)$^{***, \$\$\$\$}$
	n = 10	n = 16)	n = 21
CXCL8 (pg/ml)	3.2 (2.0 – 11.5)	3.3 (1.4 – 9.3)	747.2 (117.6 – 4986)$^{****, \$\$\$\$}$
	n = 10	n = 16	n = 21
GM-CSF (pg/ml)	13.9 (9.3 – 17.2)	12.2 (6.7 – 16.2)	36.4 (14.5 – 110.9)$^{*, \$\$}$
	n = 5	n = 8	n = 13
M-CSF (pg/ml)	< 0.5	< 0.5	1.2 (< 0.5 – 3.5)$^{*, \$}$
	n = 5	n = 8	n = 8
G-CSF (pg/ml)	10.3 (7.3 – 75.8)	8.4 (4.2 – 17.8)	8.8 (1.7 – 25.8)

n =5 n = 8 n = 13

Multiplex technology was used to measure cytokine concentrations in plasma samples from healthy controls (PedCo) and JIA patients (JIA) and in synovial fluids from JIA patients (JIA SF). Results are represented as median (range) and were statistically analyzed by Kruskal-Wallis with Dunn's multiple comparisons tests. * p 0.05; ** p 0.01; *** p 0.001; **** p 0.0001 for statistical differences between PedCo and other groups. $ p 0.05;$$ p 0.01;$$$ p 0.001;$$$$ p 0.0001 for statistical differences between JIA and other groups. Abbreviations: IFN-, interferon; IL-, interleukin-; TNF-, tumor necrosis factor.

Acknowledgements

The authors thank all patients and healthy volunteers and their legal representatives.

Contributors

J.Y., C.W. and L.D.S. were responsible for diagnosis and recruitment of patients. M.M., B.M.D., K.Y. and S.V. performed experiments and analyzed data under supervision of P.P., P.M., C.W. and L.D.S. M.M., B.M.D., P.M., P.P., C.W. and L.D.S. were involved in study conceptualization and design. M.M. wrote the initial manuscript which was critically revised by all other authors. All authors approved the final version of the manuscript.

Conflicts of interests

C.W. obtained unrestricted grants to KU Leuven from Novartis, Roche, GSK immuno-inflammation and Pfizer.

References

1. Leong JY, Guan YJ, Albani S, Arkachaisri T. Recent advances in our understanding of the pathogenesis of juvenile idiopathic arthritis and their potential clinical implications. Expert Rev *Clin Immunol* 2018;14:933–944.

2. Lin Y-T, Wang C-T, Gershwin ME, Chiang B-L. The pathogenesis of oligoarticular/polyarticular vs systemic juvenile idiopathic arthritis. Autoimmun Rev 2011;10:482–489.

3. Macaubas C, Nguyen K, Milojevic D, Park JL, Mellins ED. Oligoarticular and polyarticular JIA: epidemiology and pathogenesis. Nat Rev Rheumatol 2009;5:616–626.

4. Wedderburn LR, Robinson N, Patel A, Varsani H, Woo P. Selective recruitment of polarized T cells expressing CCR5 and CXCR3 to the inflamed joints of children with juvenile idiopathic arthritis. Arthritis Rheum 2000;43:765–774.

5. Hinks A, Cobb J, Marion MC, Prahalad S, Sudman M, Bowes J, et al. Dense genotyping of immune-related disease regions identifies 14 new susceptibility loci for juvenile idiopathic arthritis. Nat Genet 2013;45:664–669.

6. Jarvis JN, Petty HR, Tang Y, Frank MB, Tessier PA, Dozmorov I, et al. Evidence for chronic, peripheral activation of neutrophils in polyarticular juvenile rheumatoid arthritis. Arthritis Res *Ther* 2006;8:R154.

7. Jarvis JN, Jiang K, Frank MB, Knowlton N, Aggarwal A, Wallace CA, et al. Gene expression profiling in neutrophils from children with polyarticular juvenile idiopathic arthritis. Arthritis *Rheum* 2009;60:1488–1495.

8. Hu Z, Jiang K, Frank MB, Chen Y, Jarvis JN. Modeling transcriptional rewiring in neutrophils through the course of treated juvenile idiopathic arthritis. Sci Rep 2018;8:7805.

9. Foell D, Wittkowski H, Hammerschmidt I, Wulffraat N, Schmeling H, Frosch M, et al. Monitoring neutrophil activation in juvenile rheumatoid arthritis by S100A12 serum concentrations. Arthritis Rheum 2004;50:1286–1295.

10. Liew PX, Kubes P. The neutrophil's role during health and disease. Physiol Rev 2019;99:1223–1248.

11. Lefkowitz DL, Gelderman MP, Fuhrmann SR, Graham S, Starnes JD 3rd, Lefkowitz SS, et al. Neutrophilic myeloperoxidase-macrophage interactions perpetuate chronic inflammation associated with experimental arthritis. Clin Immunol 1999;91:145–155.

12. Pereira HA, Shafer WM, Pohl J, Martin LE, Spitznagel JK. CAP37, a human neutrophil-derived chemotactic factor with monocyte specific activity. J Clin Invest 1990;85:1468–1476.

13. Bennouna S, Denkers EY. Microbial antigen triggers rapid mobilization of TNF-alpha to the surface of mouse neutrophils transforming them into inducers of high-level dendritic cell TNF-alpha production. J Immunol 2005;174:4845–4851.

14. Scapini P, Carletto A, Nardelli B, Calzetti F, Roschke V, Merigo F, et al. Proinflammatory mediators elicit secretion of the intracellular B-lymphocyte stimulator pool (BLyS) that is stored in activated neutrophils: implications for inflammatory diseases. Blood 2005;105:830–837.

15. Ethuin F, Gerard B, Benna JE, Boutten A, Gougereot-Pocidalo M-A, Jacob L, et al. Human neutrophils produce interferon gamma upon stimulation by interleukin-12. Lab Invest 2004;84:1363–1371.

16. Silvestre-Roig C, Hidalgo A, Soehnlein O. Neutrophil heterogeneity: implications for homeostasis and pathogenesis. Blood 2016;127:2173–2181.

17. Headland SE, Norling L V. The resolution of inflammation: principles and challenges. Semin *Immunol* 2015;27:149–160.

18. Metzemaekers M, Gouwy M, Proost P. Neutrophil chemoattractant receptors in health and disease: double-edged swords. Cell Mol Immunol 2020;17:433-450.

19. Sandilands GP, Ahmed Z, Perry N, Davison M, Lupton A, Young B. Cross-linking of neutrophil CD11b results in rapid cell surface expression of molecules required for antigen presentation and T-cell activation. Immunology 2005;114:354–368.

20. Matsushima H, Geng S, Lu R, Okamoto T, Yao Y, Mayuzumi N, et al. Neutrophil differentiation into a unique hybrid population exhibiting dual phenotype and functionality of neutrophils and dendritic cells. Blood 2013;121:1677–1689.

21. Tak T, Wijten P, Heeres M, Pickkers P, Scholten A, Heck AJR, et al. Human CD62L(dim) neutrophils identified as a separate subset by proteome profiling and in vivo pulse-chase labeling.

Blood 2017;129:3476–3485.

22. Arebro J, Ekstedt S, Hjalmarsson E, Winqvist O, Kumlien Georén S, Cardell L-O. A possible role for neutrophils in allergic rhinitis revealed after cellular subclassification. Sci Rep 2017;7:43568.

23. Zhang D, Chen G, Manwani D, Mortha A, Xu C, Faith JJ, et al. Neutrophil ageing is regulated by the microbiome. Nature 2015;525:528–532.

24. Fortunati E, Kazemier KM, Grutters JC, Koenderman L, Bosch van JMM Van den. Human neutrophils switch to an activated phenotype after homing to the lung irrespective of inflammatory disease. Clin Exp Immunol 2009;155:559–566.

25. Sengelov H, Follin P, Kjeldsen L, Lollike K, Dahlgren C, Borregaard N. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J Immunol 1995;154:4157–4165.

26. Kadioglu A, Filippo K De, Bangert M, Fernandes VE, Richards L, Jones K, et al. The integrins Mac-1 and alpha4beta1 perform crucial roles in neutrophil and T cell recruitment to lungs during Streptococcus pneumoniae infection. J Immunol 2011;186:5907–5915.

27. Woodfin A, Beyrau M, Voisin M-B, Ma B, Whiteford JR, Hordijk PL, et al. ICAM-1-expressing neutrophils exhibit enhanced effector functions in murine models of endotoxemia. *Blood* 2016;127:898–907.

28. Kamphuis S, Hrafnkelsdottir K, Klein MR, Jager W de, Haverkamp MH, Bilsen JHM van, et al. Novel self-epitopes derived from aggrecan, fibrillin, and matrix metalloproteinase-3 drive distinct autoreactive T-cell responses in juvenile idiopathic arthritis and in health. Arthritis Res *Ther* 2006;8:R178.

29. Sedlackova L, Velek J, Vavrincova P, Hromadnikova I. Peripheral blood mononuclear cell responses to heat shock proteins and their derived synthetic peptides in juvenile idiopathic arthritis patients. Inflamm Res 2006;55:153–159.

30. Graeff-Meeder ER De, Zee R van der, Rijkers GT, Schuurman HJ, Kuis W, Bijlsma JW, et al. Recognition of human 60 kD heat shock protein by mononuclear cells from patients with juvenile chronic arthritis. Lancet (London, England) 1991;337:1368–1372.

31. Massa M, Passalia M, Manzoni SM, Campanelli R, Ciardelli L, Yung GP, et al. Differential recognition of heat-shock protein dnaJ-derived epitopes by effector and Treg cells leads to modulation of inflammation in juvenile idiopathic arthritis. Arthritis Rheum 2007;56:1648–1657.

32. Kleer IM de, Kamphuis SM, Rijkers GT, Scholtens L, Gordon G, Jager W De, et al. The spontaneous remission of juvenile idiopathic arthritis is characterized by CD30+ T cells directed to human heat-shock protein 60 capable of producing the regulatory cytokine interleukin-10. *Arthritis* Rheum 2003;48:2001–2010.

33. Nguyen TTH, Gehrmann M, Zlacka D, Sosna A, Vavrincova P, Multhoff G, et al. Heat shock protein 70 membrane expression on fibroblast-like synovial cells derived from synovial tissue of patients with rheumatoid and juvenile idiopathic arthritis. Scand J Rheumatol 2006;35:447–453.

34. Haar NM Ter, Tak T, Mokry M, Scholman RC, Meerding JM, Jager W de, et al. Reversal of sepsis-like features of neutrophils by interleukin-1 blockade in patients with systemic-onset juvenile idiopathic arthritis. Arthritis Rheumatol (Hoboken, NJ) 2018;70:943–956.

35. Pillay J, Kamp VM, Hoffen E van, Visser T, Tak T, Lammers J-W, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. J Clin *Invest* 2012;122:327–336.

36. Casanova-Acebes M, Pitaval C, Weiss LA, Nombela-Arrieta C, Chevre R, A-Gonzalez N, et al. Rhythmic modulation of the hematopoietic niche through neutrophil clearance. Cell 2013;153:1025–1035.

37. Tanji-Matsuba K, Eeden SF van, Saito Y, Okazawa M, Klut ME, Hayashi S, et al. Functional changes in aging polymorphonuclear leukocytes. Circulation 1998;97:91–98.

38. Bruhl H, Wagner K, Kellner H, Schattenkirchner M, Schlondorff D, Mack M. Surface expression of CC- and CXC-chemokine receptors on leucocyte subsets in inflammatory joint diseases. Clin Exp Immunol 2001;126:551–559.

39. Rankin SM. The bone marrow: a site of neutrophil clearance. J Leukoc Biol 2010;88:241–251.

40. Uhl B, Vadlau Y, Zuchtriegel G, Nekolla K, Sharaf K, Gaertner F, et al. Aged neutrophils contribute to the first line of defense in the acute inflammatory response. Blood 2016;128:2327–2337.

41. Sandilands GP, McCrae J, Hill K, Perry M, Baxter D. Major histocompatibility complex class II (DR) antigen and costimulatory molecules on in vitro and in vivo activated human polymorphonuclear neutrophils. Immunology 2006;119:562–571.

42. Samadi N, Polak D, Kitzmuller C, Steinberger P, Zlabinger GJ, Jahn-Schmid B, et al. T-cell-derived cytokines enhance the antigen-presenting capacity of human neutrophils. Eur J Immunol 2019;49:1441–1443.

43. Fanger NA, Liu C, Guyre PM, Wardwell K, O'Neil J, Guo TL, et al. Activation of human T cells by major histocompatability complex class II expressing neutrophils: proliferation in the presence of superantigen, but not tetanus toxoid. Blood 1997;89:4128–4135.

44. Ashtekar AR, Saha B. Poly's plea: membership to the club of APCs. Trends Immunol 2003;24:485–490.

45. Radsak M, Iking-Konert C, Stegmaier S, Andrassy K, Hansch GM. Polymorphonuclear neutrophils as accessory cells for T-cell activation: major histocompatibility complex class II restricted antigen-dependent induction of T-cell proliferation. Immunology 2000;101:521–530.

46. Ode Y, Aziz M, Wang P. CIRP increases ICAM-1(+) phenotype of neutrophils exhibiting elevated iNOS and NETs in sepsis. J Leukoc Biol 2018;103:693–707.

Figure legends

Figure 1 Analysis of neutrophil maturation and segmentation. Maturation characteristics of blood neutrophils from pediatric controls (PedCo) and JIA patients (JIA) and synovial fluid neutrophils (JIA SF) were assessed based on CD16 and CD62L expression using flow cytometry. Pleural fluid neutrophils from patients with pleural effusions (PF) and blood neutrophils from healthy adults (AdCo) were investigated for comparative purposes. Relative percentages of $CD16^{high}CD62L^{high}$ (A), $CD62L^{low}$ (B) and $CD16^{med}CD62L^{high}$ (C) neutrophils were determined. Relative percentages of CXCR4-positive aged neutrophils are shown in panel D. Blood neutrophils from healthy donors were exposed to cell-free plasma from the corresponding donor or patient synovial fluids, with or without 2 µg/ml (ethanercept, Eth), (anakinra, Ana) and/or anti-IL-6 (tocilizumab, Toc) during 24 hours and the presence of $CD62L^{low}$ (E) and $CXCR4^{+}$ (F) neutrophils was determined. Patients with oligoarticular or polyarticular disease are indicated with closed or open circles, respectively. Horizontal lines and bars indicate median values. Results were analyzed by Kruskal-Wallis with Dunn's multiple comparisons tests. * $p \leq 0.05$; ** p 0.01; *** p 0.001; **** p 0.0001.

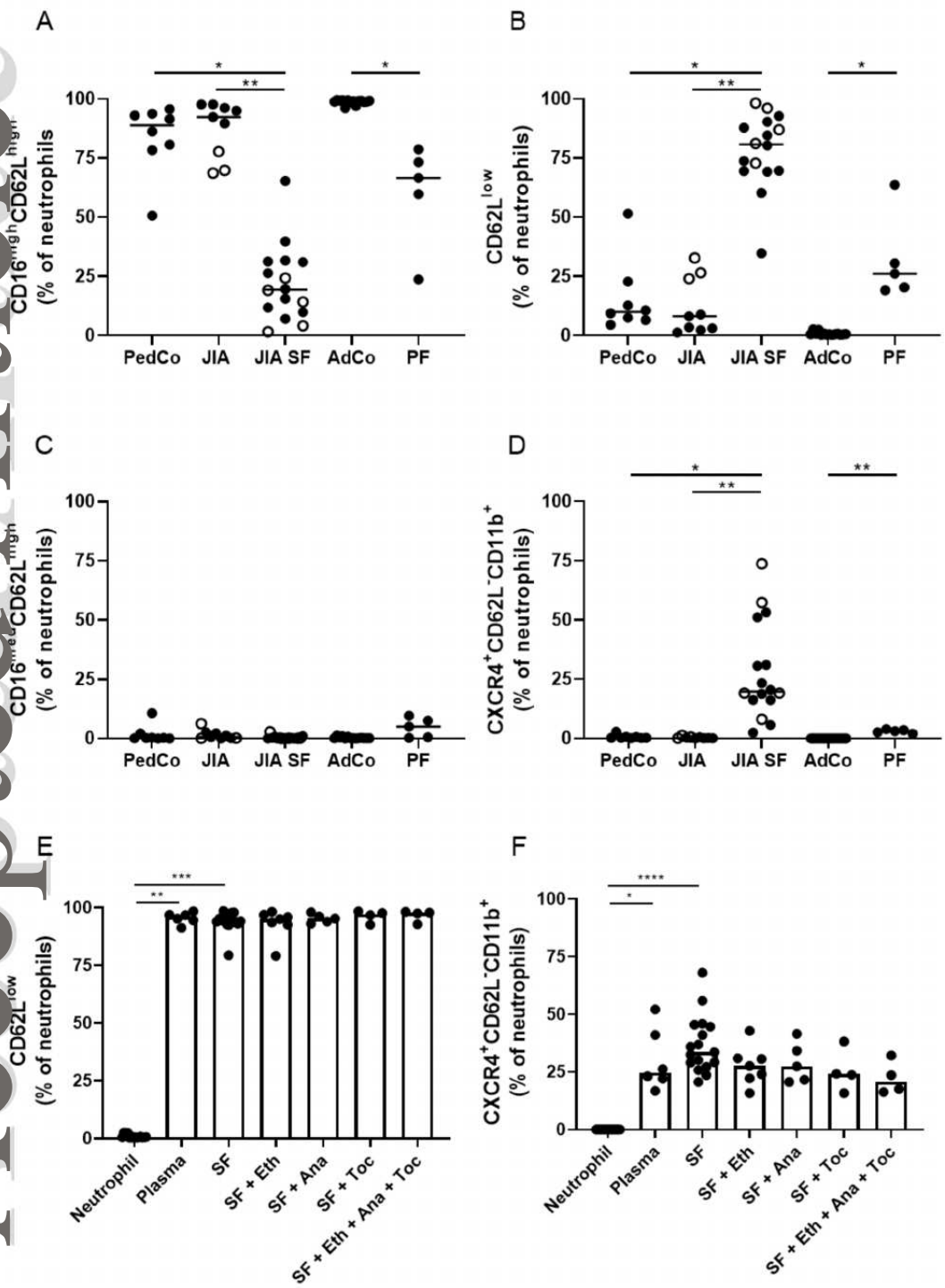
Figure 2 Analysis of adhesion molecule and HLA-DR expression. Expression of CD66b (A), HLA-DR (B), CD11b (C), CD15 (D) and ICAM-1 (E, left panel) on neutrophils present in blood from healthy pediatric controls (PedCo) and JIA patients (JIA), or in patient synovial fluids (JIA SF) were determined by flow cytometry. Pleural neutrophils from patients with pleural effusion (PF) and blood neutrophils from adult healthy volunteers (AdCo) were investigated for comparative purposes. Fresh peripheral blood neutrophils from healthy donors were exposed to cell-free plasma from the corresponding donor or cell-free synovial fluids from JIA patients, with or without 2 µg/ml (ethanercept, Eth), (anakinra, Ana) a (tocilizumab, Toc) during 24 hours, followed by analysis of ICAM-1 expression (E, right panel). Results represent percentages of positive neutrophils or mean fluorescence intensity (MFI). Patients with oligoarticular or polyarticular disease are indicated with closed or open circles, respectively. Horizontal lines and bars indicate median values. Results were statistically analyzed by Kruskal-Wallis with Dunn's multiple comparisons tests. * p 0.05; ** p 0.01; **** p 0.0001.

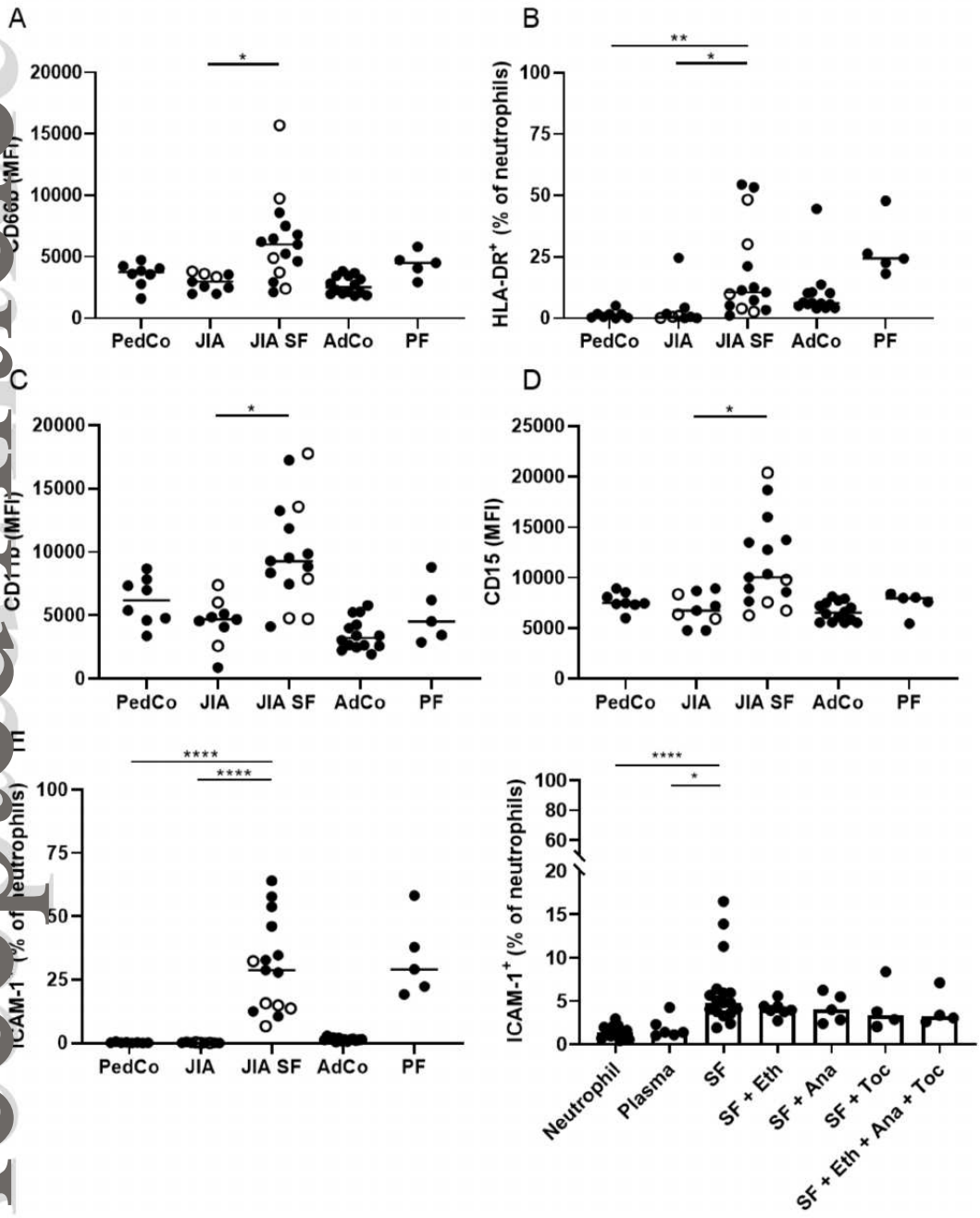
Figure 3 Analysis of CXCR1, CXCR2 and CXCR3 expression. Expression of CXCR1 (A), CXCR2 (B) and CXCR3 (C) on neutrophils present in blood from healthy pediatric controls

(PedCo) and JIA patients (JIA), or in patient synovial fluids (JIA SF) were determined by flow cytometry (left part of the figure). Pleural neutrophils from patients with pleural effusion (PF) and blood neutrophils from adult healthy volunteers (AdCo) were investigated for comparative purposes. Fresh peripheral blood neutrophils from healthy donors were exposed to cell-free plasma from the corresponding donor or cell-free synovial fluids from JIA patients, with or without 2 µg/ml (ethanercept, Eth), (anakinra, Ana) a ocilizumab, Toc) during 24 hours, followed by analysis of CXCR1 (A), CXCR2 (B) and CXCR3 (C) expression (right part of the figure). Results represent percentages of positive neutrophils or mean fluorescence intensity (MFI). Patients with oligoarticular or polyarticular disease are ndicated with closed or open circles, respectively. Horizontal lines and bars median values. Results were statistically analyzed by Kruskal-Wallis with Dunn's multiple comparisons tests. * p 0.05; ** p 0.01; *** p 0.001.

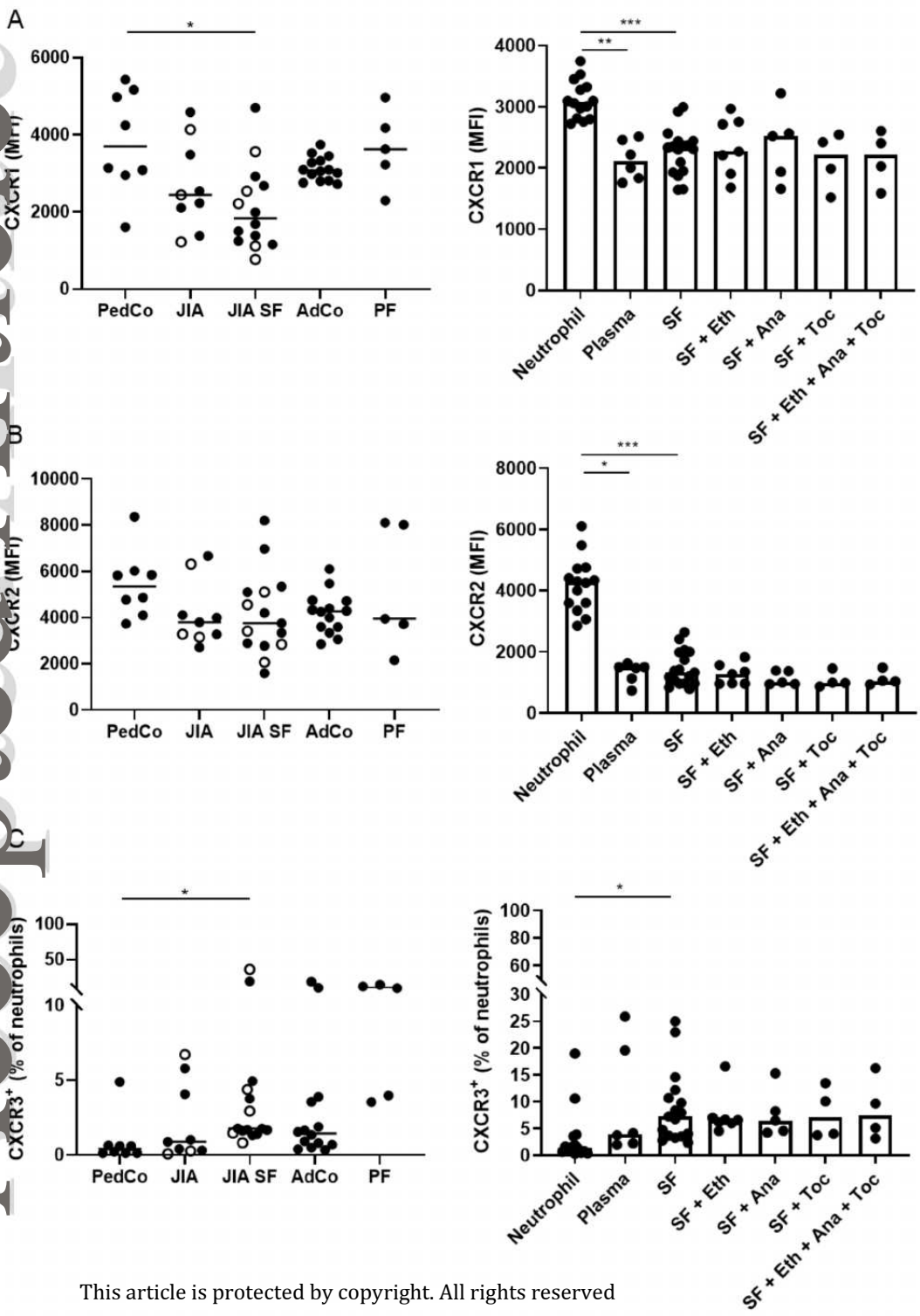
Figure 4 Analysis of C5aR, FPR1 and BLTR1 expression. Expression of C5aR (A), FPR1 (B) and BLTR1 (C) on neutrophils present in blood from healthy pediatric controls (PedCo) and JIA patients (JIA), or in patient synovial fluids (JIA SF) were determined by flow cytometry (left part of the figure). Pleural neutrophils from patients with pleural effusion (PF) and blood neutrophils from adult healthy volunteers (AdCo) were investigated for comparative purposes. Fresh peripheral blood neutrophils from healthy donors were exposed to cell-free plasma from the corresponding donor or cell-free synovial fluids from JIA patients, with or without 2 µg/ml anti-NF-α ((ethanercept, Eth), (anakinra, Ana) and/or anti-IL-6 (tocilizumab, Toc) 24 hours, followed by analysis of C5aR (A), FPR1 (B) and BLTR1 (C) expression (right part of the figure). Results represent mean fluorescence intensity (MFI). Patients with oligoarticular or polyarticular disease are indicated with closed or open circles, respectively. Horizontal lines and bars indicate median values. Results were statistically analyzed by Kruskal-Wallis with Dunn's multiple comparisons tests. * p 0.05; ** p 0.01; **** p 0.0001.

Figure 5 Correlation between phenotypical features of synovial neutrophils and local cytokine levels. Spearman correlation coefficients were calculated to detect potential correlations between the abundance of synovial cytokines and phenotypical features of synovial neutrophils om JIA patients. p values lower than the Bonferroni-corrected alpha value were considered significant. *p 0.002; $ p 0.0004; # p 0.00004. X, not determined.

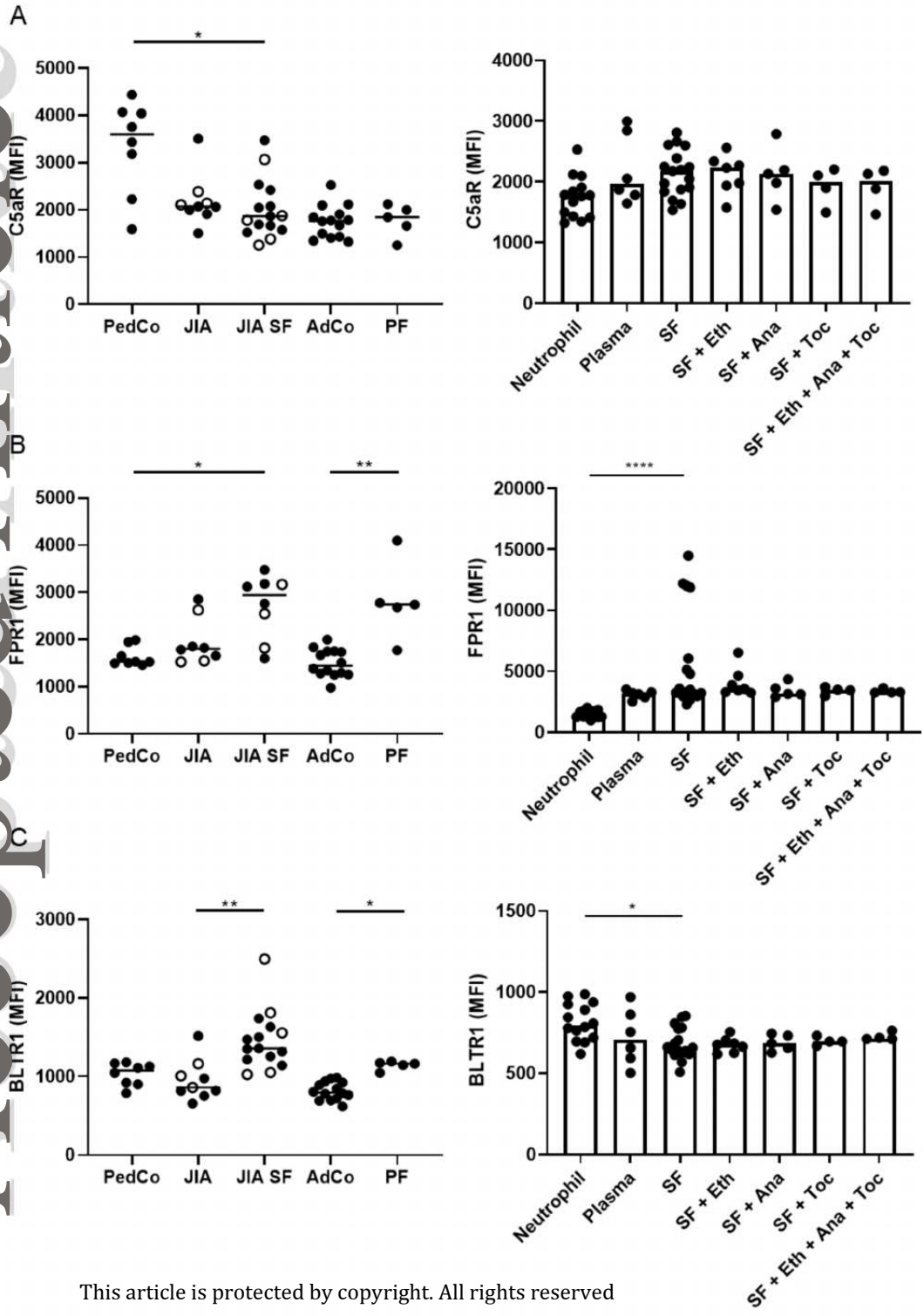




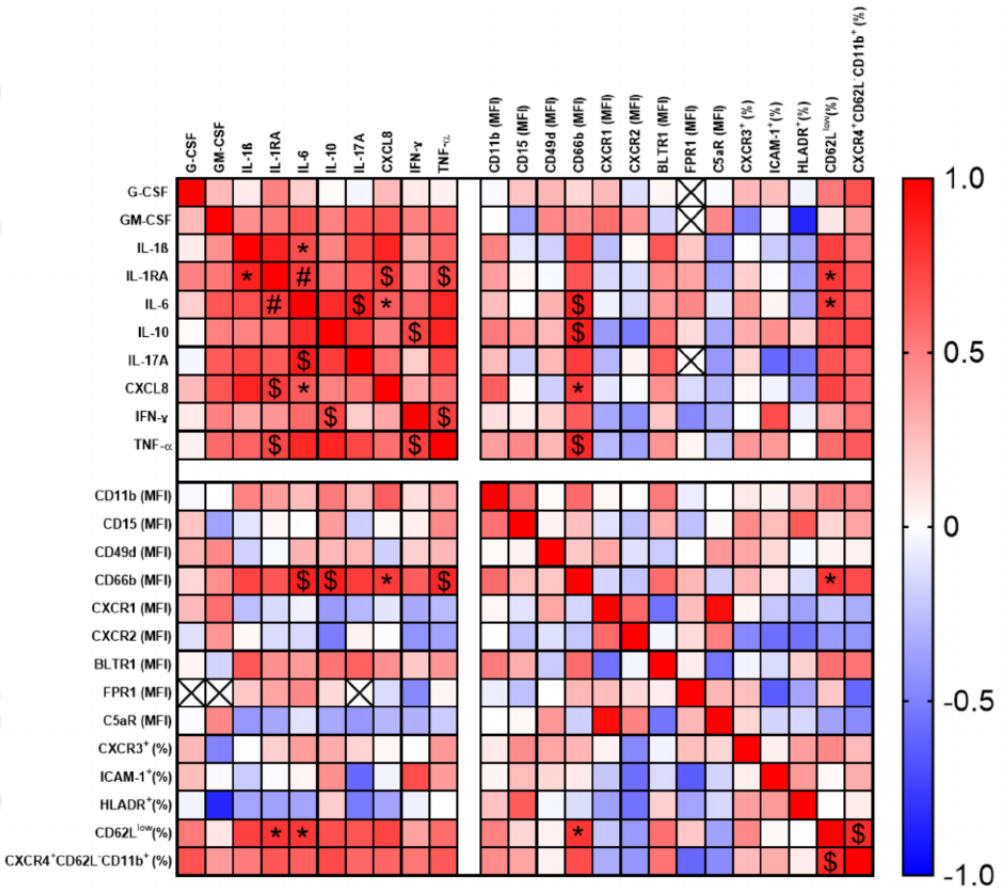
art_41605_f2.tiff



art_41605_f3.tiff



art_41605_f4.tiff



art_41605_f5.tiff