Neutrophil activation signature in juvenile idiopathic arthritis indicates the presence of low density granulocytes

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Key messages: 1) Low Density Granulocytes are elevated in JIA and contribute to transcriptomic profile of JIA PBMCs. 2) Neutrophils from JIA patients have elevated transcription of genes encoding for granule proteins. 3) The neutrophil activation signature described has important potential implications for disease pathology in JIA.

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ABSTRACT

Objective: Juvenile Idiopathic Arthritis (JIA) is an autoimmune, inflammatory disease with involvement of innate and adaptive immune responses. However, the role of neutrophils in JIA pathogenesis remains unclear. This study aimed to identify and validate neutrophil gene expression signatures in JIA using public microarray datasets and new clinical samples.

Methods: Three suitable datasets were analysed by significance analysis of microarray (SAM) and Ingenuity. Neutrophils and PBMCs were isolated from a new cohort of JIA patients and healthy paediatric controls (HC). Gene expression was validated using qPCR. Serum concentrations of proteins were measured using ELISA. Low density granulocytes (LDGs) in JIA and HC PBMCs were quantified by flow cytometry using forward/side-scatter properties.

Results: Ingenuity identified transcriptional regulation (FDR <0.05) by G-CSF, GM-CSF and IL-8 along with expression of neutrophil granule protein genes including elastase, myeloperoxidase, MMP8 and MMP9 in datasets from JIA PBMCs. LDG counts were elevated in JIA compared to HC (2.5% vs 1.4% p=0.007). Transcripts for MMP8 (p=0.005), MPO (p=0.0124) and FCGR1B (p=0.0417) were significantly higher in JIA compared to HC neutrophils. MMP9 protein levels were lower in sJIA (355.95ng/ml ±250.03ng/ml) patient sera compared to HC (675.41ng/ml ± 181.17ng/ml; p=0.007), but levels of elastase, myeloperoxidase and MMP8 were not significantly different.

Conclusion: LDGs are elevated in JIA and contribute to the transcriptomic profile of JIA PBMCs. JIA neutrophils express higher levels of MMP8 and FCGR1B which may be implicated in disease pathology through the release of proteases and reactive oxygen metabolites, which cause systemic inflammation and damage to joints.
INTRODUCTION

Juvenile Idiopathic Arthritis (JIA) is a leading cause of rheumatic disease in childhood, encompassing a heterogeneous group of conditions characterised by joint inflammation of unknown origin persisting for over a period of six weeks[1, 2]. Presentation occurs in early childhood before the age of sixteen. JIA has been reported as one of the leading causes of both long and short-term disability amongst children, affecting approximately 10 in every 100,000 children[1, 2]. The International League of Associations for Rheumatology (ILAR) classifies JIA into a number of clinical subtypes, including polyarticular (pJIA); oligoarticular (oJIA), systemic (sJIA), enthesitis related JIA, psoriatic JIA and undifferentiated arthritis [3, 4]. Both pJIA and oJIA are the predominant forms, accounting for over half of presenting cases within the UK with patients [5]. While all subtypes of JIA tend to present with a clinical manifestation of localised inflammation in the joints, sJIA is also characterised by a range of extra-articular features, including fever, rash, and hepatosplenomegaly, many of which have been associated with growth disorders leading to disability amongst patients[6]. sJIA is considered a more acute and severe subtype of JIA, and it is estimated that approximately 10% of individuals suffering from sJIA progress to macrophage activation syndrome (MAS), which can be fatal[7].

The presence of CD4+ T lymphocytes showing a CD45RO+ phenotype in inflamed synovium[8], combined with the association of Human Leukocyte Antigen (HLA) variants to disease risk of JIA[9], strongly supports the adaptive immune response in the pathogenesis of JIA[10-12]. The role of the innate immune system, and in particular neutrophils, in JIA pathogenesis is less clear despite the fact that neutrophils are found at high concentrations in JIA synovial fluid[5, 13, 14]. Activated neutrophils play a key role in the pathogenesis of
adult and juvenile autoimmune inflammatory diseases (including rheumatoid arthritis (RA) and juvenile-onset systemic lupus erythematosus; JSLE) via their ability to release reactive oxygen species (ROS), proteases and cytokines, driving the inflammatory process and causing damage to host tissues[15-17]. Neutrophils are the most abundant cells within JIA synovial fluids, and neutrophil-derived proteases and cytokines, including myeloperoxidase (MPO), neutrophil elastase (NE), matrix metalloproteases (MMPs) and interleukin-8 (IL-8), have been found at high concentrations at sites of inflammation in adult inflammatory arthritis[18-20]. In adult RA, the release of MMP8, MMP9 and NE from neutrophils at the pannus-cartilage interface can cause degradation of the collagen matrix[18, 19]. In sJIA, levels of neutrophil-derived calgranulins S100A8/9 and S100A12 serve as serum biomarkers for the diagnosis of JIA, and correlate with disease activity[21]. pJIA neutrophils demonstrate disruption of gene regulatory networks in clusters of genes modulated by IL-8 and interferon-gamma (IFN-γ)[14], with pJIA neutrophils remaining in an activated state even during periods of disease quiescence[14].

This study hypothesized that neutrophil gene expression signatures would be significantly different between JIA patients in comparison to healthy paediatric controls, and that different gene expression profiles may be apparent across JIA subtypes. The aim of this study was to identify neutrophil gene expression signatures from public microarray datasets, and to then validate these gene expression profiles in a new cohort of JIA patients. Here we report identification of neutrophil activation signatures in JIA peripheral blood. These include expression of neutrophil granule protein genes in PBMCs, elevated numbers of low density granulocytes (LDGs) in peripheral blood and increased expression of granule protein genes, interleukin-8 and FcγR1B in JIA neutrophils.
MATERIAL AND METHODS

Study population
The study was approved by North West – Liverpool East Research Ethics Committee. The study formed part of the “UK JSLE Cohort Study & Repository” (REC: 6/Q1502/77), and written informed patient/parental assent/consent obtained in accordance with the declaration of Helsinki. All JIA and healthy control patients were recruited in the study at Alder Hey Children’s NHS Foundation Trust in Liverpool, from either outpatient clinics or inpatient wards. JIA patients fulfilled the validated criteria endorsed by the International League of Associations of Rheumatology, before the age of seventeen[3]. Standard clinical data to determine disease activity/current disease state was collected for each of the JIA patients. Paediatric healthy controls had no personal or direct family history of rheumatic or chronic inflammatory disease, or acute or recent history of infection. Clinical demographics for each patient cohort are detailed in Supplementary Table 1. Due to the limited volumes of blood available from paediatric patients, we were unable to use the same patient samples for each set of experiments, and as such the number of patients analysed within JIA subtypes differs for some experiments.

Identifying datasets from public repositories
Suitable microarray datasets were selected from public repositories: NCBI Gene Expression Omnibus (GEO) database and EMBL-EBI Array Express. Key phrases such as “juvenile idiopathic arthritis” and “children” were used when performing searches identifying 20 relevant datasets in GEO and 17 in Array Express. Of these, datasets that had no data on systemic juvenile idiopathic arthritis (sJIA) or duplicates datasets between GEO and Array express, as well as those that had patients currently on treatment, were manually eliminated, providing 2 datasets that could be utilised; E-GEOD-13501[22] and GSE21521[23]. However, we noted that the samples for both datasets came from peripheral
blood mononuclear cells (PBMC’s) and due to uncertainty of whether a neutrophil signature would be found in PBMC’s, an additional dataset which contained samples that were collected from whole blood was also added to the list, GSE55319[24] providing an overall total of 3 datasets (Supplementary Table 2).

**Analysis of Microarray datasets**

Significance analysis of microarrays (SAM) was carried out on the datasets using Multi Experiment Viewer (MeV) v4[25] to identify any significantly different gene expression (False Discovery Rate adjusted p-value<0.05). Hierarchical cluster analysis was performed in MeV using Euclidean clustering and average linkage.

**Bioinformatics Analysis**

Bioinformatics analysis of differentially expressed genes (DEG) was carried out using Ingenuity Pathway Analysis (IPA). All significant DEG from each data set that could be associated with a canonical pathway within Ingenuity knowledge base were analysed, as previously described[26, 27].

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Archived serum samples from 24 JIA patients (sJIA, n=12 and pJIA, n=12) and 12 paediatric healthy controls were analysed by ELISA and age and sex matched where possible. Auto-antibodies (which may interfere with ELISA antibodies and cause false-positive results) were removed by incubating sera in a 96-well plate coated with protein L for 60 minutes (ThermoFisher Scientific, Paisley, UK) prior to assay[28]. The concentration of each of the proteases was determined using commercially available ELISA kits (purchased from R&D systems, Abingdon, UK unless otherwise stated) and protocols provided by the manufacturer: NE (PMN Elastase Human ELISA kit, Abcam, Cambridge, UK); MPO; MMP8;
MMP9. The serum concentrations of each of the detected proteins were calculated with reference to the standard curve, and expressed as nanograms per milliliter (ng/mL).

**Cell preparation**

Fresh samples of heparinized blood were used to isolate neutrophils (purity >95%). Briefly, at room temperature, whole blood was mixed for 30 mins with HetaSep™ (Stemcell, Cambridge, UK) at a 5:1 ratio in order to sediment red blood cells. The resulting leukocyte-rich buffy coat was removed and layered onto Histopaque® (Sigma, Gillingham, UK), and centrifuged at 800g for 20 minutes. Separated neutrophils and PBMCs were washed and re-suspended in RPMI media. Any contaminating erythrocytes within the neutrophil pellet were removed using hypotonic lysis, and cells were resuspended in RPMI media (Life Technologies, Paisley, UK). An aliquot of neutrophils and PBMCs were centrifuged at 500g for five minutes onto a microscope slide (cytospin) to which Romansky staining (TCS Biosciences, Botolph Claydon, UK) was applied, enabling confirmation of purity and morphology under a light microscope. Isolated neutrophils and PBMCs were analysed by flow cytometry (Beckman coulter FC500 MPL) to determine cell purity, and the presence of LDGs within the PBMC population. 10,000 events were analysed.

**RNA extraction and qPCR**

RNA was extracted from freshly-isolated neutrophils from fifteen JIA (sJIA n=2, pJIA n=7, oJIA n=4, other JIA n=3) and ten HC patients, and PBMCs from 6 JIA (sJIA, n=3, pJIA n=1, other JIA n=2) and 4 HC patients using Trizol:chloroform precipitation (Life Technologies, Paisley, UK), followed by clean-up using the RNeasy® Mini Kit which included a DNase digestion step (Qiagen, Manchester, UK). RNA from each sample was reverse-transcribed into cDNA using the Affinity Script cDNA Synthesis kit (Qiagen, Manchester, UK). qPCR was performed using the Brilliant ultra-fast SYBR® green qPCR mastermix (Qiagen,
Manchester, UK) and validated primers (Supplementary Table 3) for candidate genes; MPO, MMP8, MMP9, NE, IL8 and FCGR1B as well the housekeeping gene, β-actin (ACTB). A 1 in 5 dilution was used on all DNA samples. PCR reactions took place in MX4000® Multiplex Quantitative QPCR system using standard the thermal cycling conditions as follows, 95°C for 3 minutes for 1 cycle, followed by 95°C for 30 seconds and 60°C for 1 minute for 40 cycles. The detection of the SYBR green was calculated and dissociation curve was obtained. All mRNA expression values for candidate genes were normalised to that of the housekeeping gene[29] using the ΔΔCt method[30].

Statistical analysis

Statistical analysis was carried out using GraphPad Prism® (v4). Patient and control data was tested for normality using the Kolmogorov-Smirnov test. For data comparing multiple groups, the Kruskal-Wallis test was performed, and the Mann-Whitney test when testing only two groups. Data was considered statistically significant when p<0.05.
RESULTS

Identification of neutrophil gene expression signatures in JIA microarray datasets

Significance analysis of microarrays (SAM) was carried out on each of the datasets using MultiExperiment Viewer (MeV) identifying significantly different gene expression (DEG, \( q<0.05 \)) between groups i.e. sJIA vs HC, pJIA vs HC, sJIA vs pJIA. The number of DEG up- and down-regulated in each dataset is shown in Table 1.

Using IPA®, we carried out bioinformatics analysis of DEG from each dataset to identify signalling pathways with different regulation in JIA (Figure 1A). The significant pathways that were identified as elevated in JIA included cytokine signalling pathways (IL-8, GM-CSF, TGFβ), kinase pathways (JAK/STAT, STAT3, PI3K/AKT, NFκB), pathways relating to cell migration (Actin cytoskeleton, Leukocyte extravasation, Integrins), pathways relating to production of ROS (production of NO and ROS, phospholipase C) and regulation of cell cycle checkpoints. Upstream regulator analysis was also performed; this is an algorithm used by IPA® which examines how many known targets of a transcription regulator (e.g. cytokine or transcription factor) are present in the dataset, and compares the direction of change to what is expected from the literature in order to predict likely relevant transcriptional regulators. In all datasets, granulocyte-colony stimulating factor (G-CSF/CSF3) was predicted to be regulating the global gene expression signature in JIA compared to HC (Supplementary Table 4).

Network analysis was then conducted to identify the relationships between G-CSF and the genes in each dataset. The network of G-CSF regulated genes included several genes coding for neutrophil granule protein genes, including MPO, MMP8, MMP9 (Figure 1B). Further analysis revealed high levels of expression of a number of neutrophil protease genes in sJIA patients from two of the datasets (GSE21521 and E-GEOD-13501) as shown in
Figure 1C and Table 2. The identification of neutrophil protease genes in sJIA PBMC microarray datasets was unexpected as neutrophils isolated by density gradient separation do not remain within the PBMC fraction. We hypothesised that the gene signature observed was due to the presence of Low Density Granulocytes (LDGs) in the PBMC datasets. LDGs have been described in adult and JSLE, as well as RA, as a distinct subset of neutrophils with an altered phenotype[31-33].

Identification of LDGs in peripheral blood mononuclear cell fraction of JIA blood
To determine the presence of LDGs in JIA PBMCs, we collected samples from 16 patients with JIA (subtypes, sJIA n=6, pJIA n=3, oJIA n=3 other JIA n=4 of which 6 were on biologics and 2 on steroids, See Supplementary Table 1) and 19 HC patients. Cells were analysed by flow cytometry based on forward/side-scatter properties, and those falling within the neutrophil gate were considered LDGs. Cytospins were prepared to confirm the presence of cells with neutrophil morphology within PBMC fractions of JIA blood (Figure 2A). The number of LDGs was significantly elevated in JIA PBMCs (median (IQ range); n=16, 1.95% (1.2-2.4%)) compared to HC PBMCs (n=19, 1.1% (0.4-2.0%)) (Figure 2B, p=0.007). The %LDG was compared to patient ESR, neutrophil count and disease duration; no correlation was observed (data not shown).

Measurement of neutrophil proteases in JIA sera
Transcripts for neutrophil granule proteins including NE, MPO, MMP8 and MMP9 were significantly elevated in public datasets for sJIA PBMCs. We therefore measured the level of neutrophil proteases present in the sera of JIA patients (sJIA n=12; pJIA n=12; of which 13 were on biologics, 6 on DMARDs and 12 on steroids, see table S1) compared to age and sex-matched healthy paediatric controls. There was no significant difference between the study groups (sJIA, pJIA, HC) in serum concentrations of NE (p=0.318), MMP8 (p=0.7), and
MPO (p=0.6; Figure 3). However, the levels of MMP9 were significantly different by ANOVA between the three study groups (Figure 3, p=0.013). Post-hoc analysis revealed that MMP9 was significantly lower in sJIA patients (median (IQ range); 93.38ng/ml (41.37-209.8)) compared to pJIA (267.0ng/ml (184.8-922.4), p=0.033) and HC (402.2ng/ml (273.2-977.3), p=0.007). The levels of MMP9 were not significantly different in pJIA patients compared to HC (p=0.235). We observed a positive correlation between the age of the patients and the serum levels of NE (p=0.01, Rs=0.4685) and MPO (p=0.03, Rs=0.3641).

Gene expression in JIA neutrophils

To test the hypothesis that genes for MPO, MMP8, MMP9, and NE are expressed higher in JIA compared to HC neutrophils, we isolated mRNA from 15 JIA (subtypes sJIA n=2, pJIA n=7, oJIA n=4, other JIA n=2, of which 12 were on biologics and 1 on DMARDS, see table S2) and 8 HC neutrophil samples. qPCR was carried out for each gene and normalised to ACTB. Ct values for NE were below the level of detection in JIA and HC neutrophils. Transcripts for MMP8 and MPO were significantly higher in JIA neutrophils (Median (IQ range); MMP8: (1.47x10^{-5} (1.02x10^{-5}-2.3x10^{-5})); MPO: (5.99x10^{-6} (4.98x10^{-6}-1.85x10^{-5})) compared to HC (MMP8: 2.42x10^{-6} (1.16x10^{-6}-3.17x10^{-6})), p=0.001; MPO: (1.41x10^{-6} (1.07x10^{-6}-3.51x10^{-6})), Figure 4A p=0.012). Whilst median MMP9 expression was higher in JIA (0.05 (0.03-0.12)) compared to HC (0.03 (0.02-0.05)), this did not reach statistical significance (Figure 4A p=0.1).

We also investigated the expression levels of IL-8 and FCGR1B. The IL8 and GMCSF signalling pathway, which activates expression of IL8 in neutrophils[26], were identified by IPA® as being elevated in JIA. FCGR1B was chosen as it was identified in all datasets as having significantly higher expression, and is directly activated by interferon-γ, a cytokine implicated in JIA pathophysiology[14]. Transcripts for IL-8 were expressed across a higher
range in JIA compared to HC (Median (IQ range) JIA 0.21 (0.04-0.79), HC 0.26 (0.03-0.46), p=0.437). Expression of FCGR1B was significantly higher in JIA (Median (IQ range) JIA 0.12 (0.006-0.02), HC 0.004 (0.003-0.009), p=0.04, Figure 4B). Finally, we decided to measure the expression levels of MPO and MMP8 in PBMCs isolated from 6 JIA (sJIA n=3, pJIA n=1, other JIA n=2) and 4 HC patients. Expression ranges of both genes were higher in JIA compared to HC confirming the presence of LDGs within the PBMC fraction of JIA leukocytes, although these did not reach statistical significance (Median (IQ range) MPO: JIA 0.012 (0.008-0.41), HC 0.02 (0.01-0.04), p=0.895; MMP8: JIA 0.01 (0.008-0.76), HC 0.008 (0.0009-0.02, p=0.448).
DISCUSSION

In this study we have analysed three publically available datasets to identify neutrophil gene expression signatures activated in JIA. We identified the expression of neutrophil protease genes, including NE, MPO, MMP8 and MMP9 in sJIA PBMCs, which indicates the presence of LDGs. Whilst LDGs only represent a small percentage of the total PBMC population, work by ourselves and others[31, 33] has shown that the expression level of many neutrophil granule protein genes is many times higher in LDGs compared to mature neutrophils.[33] Similar large increases in expression of neutrophil granule protein genes, including elastase and MPO, have been reported in JIA PBMCs and attributed to an innate immune signature[34]. We confirmed the presence of LDGs in JIA by analysing samples from a new cohort of JIA patients. LDG’s have been described in other rheumatic conditions, including SLE and JSLE[31, 32] and RA[33]. These studies have explored LDG functional capacity and potential to contribute to clinical manifestations. In adult SLE, LDGs contribute to a heightened pro-inflammatory responses and production of interferon (IFN)-α via increased neutrophil extracellular trap production (NETosis)[31]. In JSLE, numbers of LDGs correlate with disease activity and titres of dsDNA antibodies[32]. In RA, LDGs are less responsive to TNF-α and represent a more immature neutrophil phenotype[33]. LDGs have also been reported to play a critical role in the pathology of other adult inflammatory diseases, including vasculitis and asthma[35, 36]. The presence of LDGs in JIA raises the possibility that these cells contribute to the pathophysiology of the disease. However whilst we have confirmed the presence of LDGs in JIA peripheral blood, the functional phenotype of these cells in JIA remains to be explored.

Neutrophils release serine proteases including NE, MMP8 and MMP9 when inappropriately activated during inflammation. MMPs, a super family of proteases, engage in physiological tissue development as well as tissue destruction[37]. By processing pro-forms of cytokines
and chemokines they are able to mediate both pro- and anti-inflammatory processes. In addition, MMP9 degrades α1-antiproteinase, a potent elastase inhibitor, perpetuating the inflammatory response[37]. Regulated by tissue inhibitors of MMP’s (TIMPs), enzymes MMP8 and MMP9 are released by neutrophils following migration to the site of inflammation. Observations have shown an imbalance between TIMPs and MMP-8/9 leads to pathological tissue destruction. Indeed both MMP8 and MMP9 are found at high concentrations in synovial fluid of adult patients with RA[38, 39]. Both MMP8 and MMP9 cleave components of the collagen matrix within the joint, including collagen V, Vii and X, aggrecan, and fibronectin[40]. Surprisingly, we found that levels of neutrophil proteases in the sera of sJIA and pJIA patients were not elevated compared to healthy controls. Moreover, MMP9 levels were significantly lower in JIA patients compared to controls, and were significantly lower in sJIA compared to pJIA. There may be several explanations for this observation: firstly, the ELISA assays used in our study measured the concentration of the proteases in serum, but did not measure enzyme activity; secondly, whilst there is evidence for elevated protease levels at the site of inflammation (synovial joints) in adult RA[18, 38, 39], it is unclear whether these proteases are elevated in sera; thirdly, the results may be explained by the heterogeneous nature of JIA subtypes; finally, all JIA patients in our study were receiving medication, with many receiving biologic and/or disease-modifying therapies, and as such this places limitations on the interpretation of our data. Studies have determined that levels of neutrophil-derived proteases can be affected by anti-inflammatory therapies. A longitudinal study showed lower levels of MMP9 in a subset of pJIA patients following a year of treatment with the TNF inhibitor etanercept[41]. The blockade of cytokine and chemokine synthesis in both peripheral blood cells and synovial tissue caused by TNF inhibitor therapy leads to lower levels of serum proteases[41, 42]. Similarly, tocilizumab, a humanised, monoclonal, IL-6 receptor (IL-6R) antibody used in the treatment of sJIA, indirectly decreases levels of MMP9[43]. The use of drugs such as methotrexate in other inflammatory
conditions such as RA has been reported to reduce levels of cytokines such as IL-8 in the serum [44, 45]. Unfortunately it is not possible to comment on the effect of treatment on LDG expression in our study, due to the small sample size; only one of the patients was not on medication and the majority were receiving biologics.

The level of expression of genes for neutrophil granule proteins NE, MPO, MMP8 and MMP9 were higher in neutrophils from all JIA subtypes compared to HC, although the levels differed across the JIA patient cohort due to the heterogeneous nature of this condition. This observation could be partially explained by suppressed immune activity during therapy. However, a recent study found that gene expression in JIA neutrophils remains in state of activation despite response to treatment[46]. Mature adult neutrophils do not normally express transcripts for neutrophil granule proteins; expression of genes such as MPO and NE are down-regulated during terminal differentiation of neutrophils prior to release from the bone marrow[47]. However, expression of neutrophil granule protein genes including MPO and NE by mature neutrophils has recently been reported in a population of RA patients, and is associated with a lack of response of RA patients to TNF inhibitor therapy[48]. Elevated transcripts for NE, MPO, MMP8 and MMP9 were identified in public datasets from sJIA PBMCs, and whilst this indicates the expression of these genes by LDGs within the PBMC fraction, it gives no information on whether these genes are elevated in JIA neutrophils.

CD64 is an interferon-γ-regulated Fcγ receptor sub-class, encoded by three genes (FCGR1A, FCGR1B, FCGR1C)[49, 50]. Neutrophil FCGR1A binds monomeric IgG and is classed as a high-affinity IgG receptor. Whilst FCGR1B and FCGR1C are commonly believed to be pseudogenes[51] lacking the extracellular immunoglobulin-like domain coded by exon 3[52], a splice variant of FCGR1B has been shown to bind aggregated immune-
complexes formed by IgG[53]. A possible role for a secreted soluble FcγR1b receptor splice variant has also been proposed[52]. Transcripts for FCGR1A were elevated in all public datasets analysed in this study, but not validated by qPCR. Increased expression of the FCGR1B transcript of CD64 by JIA neutrophils has not been previously described and is reported here for the first time. In adult RA, CD64 is expressed at high amounts in neutrophils aspirated from synovial joints, and expression can be induced in HC neutrophils by the addition of interferon-γ or cell-free RA synovial fluid[54]. Neutrophils expressing the CD64 receptor can be activated by monomeric IgG to produce a respiratory burst (ROS), implicated in damage to host tissue in inflammatory diseases such as RA and COPD[15, 16, 50]. Expression of FCGR1B by neutrophils in JIA could therefore provide insight into the disease pathology, particularly in those pJIA patients who are rheumatoid factor positive. Of note, FCGR1B expression was higher in the pJIA patients when we analysed the dataset by JIA subtype. However, further investigation of CD64 protein expression is required to confirm the role of FCGR1B in the pathology of JIA.

In summary, we describe here for the first time a neutrophil activation signature in JIA patients, which is due in part to the presence of elevated numbers of LDGs. Neutrophils from JIA patients have elevated transcription of genes encoding granule proteins, including MMP8, MMP9 and MPO, as well as IL-8 and the high-affinity IgG receptor CD64. This activation signature has important potential implications for disease pathology in JIA, as the release of proteases and reactive oxygen metabolites (in response to activation of FcγRs) is a major cause of systemic inflammation and damage to joints.
ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

None declared
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TABLES

Table 1: Number of significantly up- and down-regulated genes identified in each dataset using significance analysis of microarrays (SAM) (FDR<0.05)

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<thead>
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<th>E-GEOID-13501 pJIA v HC</th>
<th>E-GEOID-13501 sJIA v pJIA</th>
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Table 2. Neutrophil granule protein genes with significant levels of gene expression in JIA microarray datasets from PBMCs (E-GEOD-13501, GSE21521) and whole blood (GSE55139) (data shown as Log2(Fold change), N.S.=not significant).

<table>
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<tr>
<th>Gene Name</th>
<th>E-GEOD-13501 sJIA v HC</th>
<th>GSE21521 sJIA v pJIA</th>
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**FIGURE LEGENDS**

**Figure 1. Ingenuity analysis of significant genes identified in public datasets for JIA.**
(A) Canonical pathways predicted to have significantly differential regulation in each dataset. All pathways predicted to have increased activation in JIA except Cell cycle: G2/M DNA damage checkpoint regulation, PTEN signaling, RhoGDI signaling, and Cell Cycle: CHK proteins checkpoint control, which were predicted to have decreased activation. (B) Network of genes regulated by G-CSF (CSF3) in JIA. Network is overlaid with expression data for dataset E-GEOD-13501 (sJIA v HC) as an example. The intensity of the red shading correlates to up-regulation of the gene in sJIA vs HC. (C) Heatmap of genes for neutrophil granule proteins that were identified as being significantly up-regulated in the datasets. Expression levels: Red=high; Black=median; Green=low.

**Figure 2. Identification of low density granulocytes (LDGs) in JIA peripheral blood.**
(A) LDGs were identified in JIA (n=16) and HC (n=19) PBMCs by forward/side scatter properties, and cell morphology on cytospin (white arrow). (B) The number of LDGs in JIA PBMCs was significantly higher than in HC PBMCs (**p=0.007**). Bars represent median ± interquartile range.

**Figure 3. Levels of neutrophil granule proteins in JIA and HC sera.** Levels of NE, MPO, MMP8 and MMP9 were measured in serum samples from age/sex-matched patients with sJIA, pJIA and HC (n=12 in each group). MMP9 levels were significantly lower in sJIA compared to HC (**p=0.007**). Bars represent median ± interquartile range.

**Figure 4. Gene expression in JIA neutrophils.** (A) The expression of genes for neutrophil granule proteins MPO (**p=0.012**), MMP8 (**p=0.001**) and MMP9 (**p=0.1**) was higher in JIA neutrophils compared to HC (JIA n=15, HC n=8). (B) Expression levels of IL8 and FCGR1B
were also higher in JIA neutrophils compared to HC (* p=0.04). Bars represent median ± interquartile range. Gene expression shown as mean normalized expression (MNE), normalized to actin (ACTB).
Figure 1
Figure 2

A

Neutrophils

PBMCs

B

% LCGs in PBMCs

JIA

Healthy

Systemic
Poly
Oligo
Other
Healthy
Median +/- quartiles
Figure 3
Figure 4

**A**

**MMP9**

- **MPO**

- **FCGR1B**

**B**

**IL8**