The role of low density granulocytes and NETosis in the pathogenesis of adult-onset Still’s disease

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ABSTRACT

Objective. To analyse the potential contribution of low-density granulocytes (LDGs) and NETosis, as well as the differential protein cargo of neutrophil extracellular traps (NETs), as physiopathogenic mechanisms of adult-onset Still’s disease (AOSD).

Methods. We recruited 30 patients with AOSD according to the Yamaguchi diagnostic criteria. LDGs were addressed by multiparametric flow cytometry as those CD14-, CD15+, CD10- cells in the peripheral blood mononuclear cells fraction. NETs were quantified by ELISA, immunofluorescence and fluorescence spectrometry. The expression of IL-37 and high mobility group box 1 (HMGB-1) in NETs was measured by immunofluorescence and confocal microscopy. Additionally, normal density neutrophils from healthy controls were stimulated with serum from patients with AOSD and NET induction was assessed by immunofluorescence.

Results. Patients with active disease as well as those with arthritis, cutaneous manifestations and fever had a higher amount of NETs and LDGs. Serum NETs from AOSD patients correlated with the number of swollen joints (r=0.41, p=0.032), absolute number of monocytes (r=0.529, p=0.005). The spontaneous NETs from patients with cutaneous manifestations and fever had higher cargo of HMGB-1 compared with patients in remission.

Conclusion. LDGs and NETs are increased in patients with active AOSD and correlate with particular clinical features. Patients with cutaneous lesions and fever present a higher cargo of HMGB1 in their spontaneous NETs.

Introduction

Adult-onset Still’s disease (AOSD) is a rare multigenic systemic autoinflammatory disorder that is considered the adult form of systemic juvenile idiopathic arthritis (JIA) (1). The main clinical manifestations of AOSD are fever, arthritis, lymphadenopathy, haematopoesis and rash (2) and during episodes of active disease acute phase reactants, such as erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and ferritin are elevated in the majority of patients (3). Frequently, this pattern of recurrent and remitting signs and symptoms implicate a challenge in the accurate approach of patients with episodic fever of unknown origin (4). The immunopathology of AOSD is characterised by an enhanced production of tumour necrosis factor-α (TNF-α), interleukin 6 (IL-6) and IL-18 (5, 6), which promote activation of macrophages and a preferential Th1 phenotype, as well as a higher production of interferon γ (IFN-γ) and IL-17 (5, 6). Additionally, increased levels of IL-18 and IL-1β, the final products of the nucleotide-binding oligomerisation (NOD) like receptor P3 (NLRP3) inflammasome pathway, are associated with increased disease activity and severity (7).

The relevance of neutrophils in the pathophysiology of AOSD is highlighted because neutrophilic leukocytosis is found in over 70% of patients during episodes of disease activity; also the neutrophil/lymphocyte ratio is associated to the diagnosis (OR 2.336, p<0.001) and correlates with the systemic score, CRP and ferritin levels (1, 2). In 2004, Brinkmann, et al. described a new form of death cell called NETosis, in which neutrophils extrude chromatin webs decorated with proteins from the cytoplasmic granules after stimuli with phorbol myristate acetate (PMA) and CXCL8 (8). These webs are named neutrophil extracellular traps (NETs), are abundant in tissue with inflammation (8) and have been reported to be relevant in the pathophysiology of many autoimmune dis-

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eases including systemic lupus erythematosus (SLE) (9), rheumatoid arthritis (RA) (10) and psoriasis (11). NETs are abundantly produced by a special subset of neutrophils named low-density granulocytes (LDGs), which had been found to be elevated in patients with SLE (12), juvenile idiopathic arthritis (13), dermatomyositis (14) and ANCA-associated vasculitis (15).

Additionally, the protein cargo of NETs is different according to diverse stimuli or disease state (16), particularly, LL-37 and high mobility group box 1 (HMGB1) are found in NETs from patients with SLE (17). Extracellular HMGB1 acts as an alarmin and induces the production of TNFα by macrophages through activation of Toll-like receptors (TLRs) and cytokine receptor signalling amplification (18), whilst the antimicrobial peptide LL-37 induces a type I IFN response by TLR-9 mediated activation of plasmacytid dendritic cells (pDC) (17). The initiation and perpetuation of an inflammatory response in AOSD seems to be mediated by an abnormal signalling through pattern recognition receptors (PRR) in neutrophils and macrophages (19). NETs are an important source of damage associated molecular patterns (DAMPs) and pro-inflammatory molecules such as DNA, histones, HMGB1 and LL-37 (20). Furthermore, NETs from patients with SLE and familial Mediterranean fever (FMF) are able to induce a differential production of cytokines in monocytes and macrophages in vitro (21, 22).

Even though, neutrophilia and an enhanced pro-inflammatory state have been widely acknowledged as critical features of AOSD, there is no data regarding the role of normal and low-density neutrophils, as well as the potential contribution of NETosis and its differential protein cargo as part of the pathophysiology of AOSD. The aim of this study was to analyse the role of LDGs and NETs in the pathogenesis of AOSD.

Material and methods
Clinical assessment of patients with AOSD
From 2016 to 2018, we recruited 30 patients with AOSD according to the Yamaguchi diagnostic criteria (23). Patients with active infection, chronic viral infections, pregnancy or puerperium at the time of evaluation were excluded. Five age and gender matched healthy controls were used as a comparison group for the evaluation of circulating LDGs, NETs and for the evaluation of the induction of NETosis with sera. Patients and controls signed an informed consent prior to inclusion and the protocol was approved by our institutional ethics and research committees (Ref. 2307) in accordance with the Helsinki declaration. Two rheumatologists (JTR and JAGG) evaluated the disease activity and active AOSD was defined as the presence of one or more of the items from the Pouchot index (24). Also, we recorded if the treating physician decided to modify or maintain the dose of immunosuppressive therapy.

Flow cytometry analysis of LDGs
Peripheral blood mononuclear cells (PBMCs) were isolated by density gradients after centrifugation with Lymphoprep (Stemcell Technologies, Vancouver, Canada). After washing twice with 5% FBS (fetal bovine serum) in PBS, PBMCs were stained with the following fluorescent labelled-antibodies: CD14-PerCP, CD10-PE, CD15-FITC (Biolegend, San Diego, CA, USA). LDGs were considered as those CD14-, CD15+, CD10+ cells in the PBMCs fraction as previously described (12, 25). Briefly, the backgating strategy of these experiments was based upon the physical properties of LDGs since they usually have the same SSC and a slightly higher FSC than normal density granulocytes (26). Once we had the selected population of CD10+, CD14- and CD15+, we made a backgating of this gate, to ensure that we had selected only cells with the size of mature neutrophils (12, 27, 28). The gating strategy is depicted in Figure 1. We expressed the percentage of LDGs taking into account the total PBMCs gate.

Induction and quantification of NETs, LL-37 and HMGB1
After density gradients, conventional neutrophils were isolated with dextran sedimentation as previously described (29). We quantified the spontaneous NET formation (without any stimuli) and lipopolysaccharide (LPS)-induced NETosis with 1 μg/mL E. coli O111:B4 LPS (Sigma Aldrich, St. Louis Missouri, USA) by fluorescence spectrometry and indirect immunofluorescence (29). Briefly, neutrophils were incubated in RPMI without phenol red (Thermo Fisher scientific), 1% FBS, and 1% 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES) at 37°C during 1.5 hrs in dark 96 well plates with 0.2 μM SYTOX green (Thermo Fisher Scientific, Waltham, Massachusetts USA). The experiments were repeated by quadruplicate and measured with a Biotek Synergy HT Spectrofluorometer (Biotek, Winoski, USA). For confocal microscopy, conventional neutrophils were seeded in 0.01% poly-L-Lysine (Sigma-Aldrich, Germany) coated coverslips at 37°C during 1.5 hrs. After fixation with 4% paraformaldehyde (Santa Cruz Biotechnology, USA) at 4°C during 24 hrs, washing and blocking with 0.02% gelatin from porcine skin (Sigma-Aldrich, Darmstadt, Germany), we performed indirect immunofluorescence using the following primary and secondary antibodies diluted in 0.02% gelatin from porcine skin: rabbit anti-human neutrophil elastase (Abcam, Cambridge, United Kingdom), mouse anti-human LL-37 (Santa Cruz Biotechnology, Dallas, Texas, USA), mouse anti human HMGB1 (Santa Cruz Biotechnology, Dallas, Texas, USA) (30), donkey anti-rabbit Alexa Fluor 555 (Thermo Fisher, Waltham, Massachusetts, USA) and donkey anti-mouse DyLight 488 (Thermo Fisher, Waltham, Massachusetts, USA). Chromatin was stained with Hoechst 33342 (Thermo Fisher, Waltham, Massachusetts, USA) and coverslips were mounted on slides with ProLong® Gold Antifade Mountant (Thermo Fisher, Waltham, Massachusetts, USA). The samples were acquired in an Eclipse Ti-E Nikon confocal microscope (Minato, Tokyo, Japan). The amount of NETs was quantified as the mean number of fibrillar structures in which chromatin co-localised with neutrophil elastase divided by the number of cells and multiplied by 100 in six 40X fields per experimental condition (spontaneous and LPS-induced) (30).
The expression of LL-37 and HMGB-1 was quantified as the mean fluorescence intensity (MFI) of DyLight Alexa fluor 488 tracing polygons around every NET excluding the cell bodies in six 40X fields per experimental condition (30). The images were analysed with the software Fiji (NIH).

Quantification of NETs by ELISA

The amount of serum DNA-neutrophil elastase (NE) complexes was measured as previously described (31). Briefly, high binding 96 well ELISA plates were incubated overnight at 4°C with rabbit anti human elastase (Abcam, Cambridge, UK) in coating buffer from the Cell Death Detection ELISA kit (Roche, Mannheim, Germany). After washing with 0.01% tween 20 (Sigma-Aldrich, Darmstadt, Germany) and blocking with 1% bovine serum albumin (BSA), plates were incubated overnight at 4°C with 200 μL of 1:10 diluted human sera in 1% BSA. After washing, we incubated the plates with the POD-anti dsDNA antibody from the Cell Death Detection ELISA during 1 hour. The plates were washed, followed by the addition of the 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Sigma, Darmstadt, Germany). Finally, we measured absorbance at 450 nm, after adding stop solution.

Induction of NETs with AOSD serum

Neutrophils from healthy controls were incubated in RPMI without phenol red and 10% serum from healthy controls and patients with AOSD during 1 hr. The amount of NETs was quantified by indirect immunofluorescence as previously described (30).

Table I. Laboratory features and characteristics of NETs of patients with AOSD according to their activity status.

<table>
<thead>
<tr>
<th>Features</th>
<th>Active Median (IQR)</th>
<th>Inactive Median (IQR)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>13.5 (11.7-14.3)</td>
<td>14.8 (14.05-16.3)</td>
<td>0.02</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>43.5 (37.2-43.1)</td>
<td>44 (42.2-48.4)</td>
<td>0.08</td>
</tr>
<tr>
<td>Total leucocyte count (x 10^9 x L)</td>
<td>11 (7.5-13.1)</td>
<td>6.9 (6.3-7.9)</td>
<td>0.045</td>
</tr>
<tr>
<td>Total neutrophil count (x 10^6 x L)</td>
<td>7881 (4899-10907)</td>
<td>4334 (3567-5237)</td>
<td>0.004</td>
</tr>
<tr>
<td>Total lymphocyte count (x 10^6 x L)</td>
<td>1974 (1059-2163)</td>
<td>1803 (1427-2329)</td>
<td>0.44</td>
</tr>
<tr>
<td>Total monocytes count (x 10^6 x L)</td>
<td>469 (217-840)</td>
<td>448 (261-523)</td>
<td>0.57</td>
</tr>
<tr>
<td>Neutrophil/lymphocyte rate</td>
<td>3.9 (2.3-10.3)</td>
<td>2.2 (1.8-3.7)</td>
<td>0.09</td>
</tr>
<tr>
<td>Platelets (x 10^11 x L)</td>
<td>300 (235-535)</td>
<td>248 (205-267)</td>
<td>0.07</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate (mm/hr)</td>
<td>21 (11.5-45)</td>
<td>5 (2.8-5)</td>
<td>0.001</td>
</tr>
<tr>
<td>C reactive protein (mg/dL)</td>
<td>6.6 (2.2-25.4)</td>
<td>0.29 (0.11-0.33)</td>
<td>0.0007</td>
</tr>
<tr>
<td>Ferritin (ng/mL)</td>
<td>366 (145-5473)</td>
<td>36 (24-49)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Alanine transaminase ALT (U/L)</td>
<td>23 (18-42.5)</td>
<td>15 (13-27.5)</td>
<td>0.04</td>
</tr>
<tr>
<td>Aspartate transaminase ALT (U/L)</td>
<td>27 (23-36.5)</td>
<td>18 (15.5-24)</td>
<td>0.019</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>117 (80-148)</td>
<td>72 (57-83)</td>
<td>0.019</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>3.9 (3-4.3)</td>
<td>4.3 (4.1-4.6)</td>
<td>0.052</td>
</tr>
<tr>
<td>Globulins (g/dL)</td>
<td>3.3 (2.6-3.6)</td>
<td>3 (2.8-3.2)</td>
<td>0.51</td>
</tr>
<tr>
<td>% Low-density granulocytes</td>
<td>2.5 (0.7-5.1)</td>
<td>0.14 (0.045-0.35)</td>
<td>0.0002</td>
</tr>
<tr>
<td>LPS1-induced NETs/2cell</td>
<td>15 (11-19)</td>
<td>18 (11-27.5)</td>
<td>0.36</td>
</tr>
<tr>
<td>Spontaneous NETs/cell</td>
<td>85 (43-246)</td>
<td>88 (68-158)</td>
<td>0.87</td>
</tr>
<tr>
<td>LPS-induced NETs area (micrometer)</td>
<td>9604 (6563-11384)</td>
<td>7705 (5289-10352)</td>
<td>0.56</td>
</tr>
<tr>
<td>Spontaneous NETs area (micrometer)</td>
<td>8625 (6650-13168)</td>
<td>7673 (4672-10412)</td>
<td>0.49</td>
</tr>
<tr>
<td>MFI1 of LL37 in LPS-induced NETs</td>
<td>124 (43-183)</td>
<td>154 (62-215)</td>
<td>0.44</td>
</tr>
<tr>
<td>MFI of LL37 in spontaneous NETs</td>
<td>85 (43-221)</td>
<td>88 (68-158)</td>
<td>0.87</td>
</tr>
<tr>
<td>MFI of HMGB14 in LPS-induced NETs</td>
<td>74 (71-165)</td>
<td>66 (37-155)</td>
<td>0.45</td>
</tr>
<tr>
<td>MFI of HMGB1 in spontaneous NETs</td>
<td>126 (38-242)</td>
<td>91 (51-122)</td>
<td>0.48</td>
</tr>
<tr>
<td>MFI of sytox green LPS</td>
<td>1268 (1299-2831)</td>
<td>1668 (828-2361)</td>
<td>0.83</td>
</tr>
<tr>
<td>MFI of sytox green spontaneous</td>
<td>1404 (1358-2876)</td>
<td>1760 (827-2276)</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Results

Clinical manifestations and NETs features of patients with AOSD

Twenty patients (66.7%) were women. The median and IQR of age was 40 (28-46) years. In Supplementary Table 1, we depict the clinical features of patients with AOSD at disease diagnosis and at the time of evaluation for this study. There were significant differences in laboratory features among active AOSD patients compared with inactive AOSD patients as previously reported in the literature (2, 4, 23, 24); those differences include lower haemoglobin (13.5 mg/dl vs. 14.8 mg/dl p=0.02), higher leucocyte count (11 x10^9/L vs 6.9 x10^9/L p=0.045), increased neutrophil count (7881 x10^9/L vs. 4334 x10^9/L p=0.004), as well as elevated erythrocyte sedimentation rate (21 mm/hr vs. 5 mm/hr p=0.001), C-reactive protein (6.6 mg/dL vs. 0.29 mg/dL p=0.0007) and ferritin levels (366 ng/ml vs. 36 ng/ml p=0.0004). Regarding the clinical course, thirteen patients (43.3%) had self-limited or monophasic, 11 (36.7%) had intermittent or polycyclic systemic and 6 (20%) had chronic articular pattern. Fourteen patients (46.7%) were on prednisone, 15 (50%) were taking methotrexate and 1 (3.3%) mycophenolate mofetil. Sixteen patients (53.3%) did not require treatment adjustment and in 5 (16.6%) the treatment was decreased. Nine patients (30%) had active disease in whom immunosuppressive therapy was augmented; 3 of them (33.3%) were receiving biologic treatment with rituximab or tocilizumab.

The laboratory features and the characteristics of the NETs from AOSD according to their disease activity status are depicted in Table I.

A higher percentage of LDGs in peripheral blood is present among patients with active AOSD

As shown in Table I and Figure 1, the percentage of LDGs was higher in patients with active disease in comparison with those with quiescent AOSD (2.5 (0.7-5.1) vs. 0.14 (0.04-0.35), p=0.0024) and with healthy controls (2.5 (0.7-5.1) vs. 0.05 (0.007-0.16),
p=0.0013). Likewise, patients with particular clinical features, such as those with both cutaneous manifestations and fever ((3.5 (1.19-7.19) vs. 0.17 (0.05-0.38), p=0.0016), and arthritis (2.11 (0.29-5.02) vs. 0.18 (0.06-0.38), p=0.034) displayed higher amount of LDGs compared to those without these manifestations.

AOSD patients have a differential concentration of NETs and their protein cargo according to the clinical features of the disease

Although we did not find a differential expression of LL-37 and HMGB1 in patients with AOSD according to their disease activity status, the median (IQR) expression of HMGB1 in spontaneous NETs from patients whom had both cutaneous manifestations and fever was higher in comparison with subjects without those clinical features (168.8 (163.6-4786), vs. 89 (43.23-122.6), p=0.025) (Fig. 2 A and Fig. 3 A-B).

Table II. Differential percentage of LDGs, concentration of NETs and their protein cargo according to the clinical features of patients with AOSD.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median (IQR) of neutrophil elastase-DNA complexes (ODI)</th>
<th>Median (IQR) of the percentage of low-density granulocytes</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arthritis</td>
<td>2.11 (0.29-5)</td>
<td>0.18 (0.06-0.38)</td>
<td>0.034</td>
</tr>
<tr>
<td>Dermatosis and fever</td>
<td>3.5 (1.19-7.19)</td>
<td>0.17 (0.05-0.38)</td>
<td>0.0016</td>
</tr>
<tr>
<td>Biologic DMARD use</td>
<td>1.23 (1.16-2.15)</td>
<td>1 (1-1.16)</td>
<td>0.028</td>
</tr>
</tbody>
</table>

1Disease modifying anti-rheumatic drugs.
Patients with global active disease as well as those with arthritis and biologic disease-modifying anti-rheumatic drugs (DMARDs) users had higher amount of circulating DNA-NE complexes (Fig. 2 B-C). We did not find a difference in the fluorescence intensity of sytox green between healthy controls and patients with AOSD (data not shown). The amount of serum NETs correlated with the number of swollen joints ($r=0.41$, $p=0.032$) and the absolute number of monocytes ($r=0.529$, $p=0.005$).

In Table II, we summarise the differential amount and protein cargo of NETs according to the clinical characteristics in AOSD patients.

**Sera from patients with active AOSD is able to induce NETosis in healthy control neutrophils without the need for previous priming**

In order to reproduce a biological approach to how the pro-inflammatory state of AOSD patients could promote the induction of NETs, healthy control neutrophils were stimulated with serum from AOSD patients. As shown in Figure 4, sera from patients with active AOSD promoted an enhanced production of NETs (Fig. 4 D-E-F) in comparison to healthy control sera (Fig. 4 A-B-C).

**Discussion**

Our data suggest that patients with active AOSD and particularly those with fever, articular and cutaneous manifestations display enhanced NETosis, with a higher amount of LDGs and circulating NETs enriched in HMGB1. LDGs are increased in patients with other diseases characterised by cutaneous and articular damage such as psoriasis, in which LDGs tended to form NETs without stimulation, in contrast to healthy control or psoriasis normal density neutrophils (32). This pattern of spontaneous NETosis is found in autoimmune/autoinflammatory diseases (33) and may result in the perpetuation of the pro-inflammatory storm seen in patients with AOSD.

Our work contributes to elucidate the role of NETs, and particularly LDGs as the link between AOSD and monogenic autoinflammatory disorders such as fa-
milial Mediterranean fever, in which NETs containing IL-1β are released during acute attacks (22) or the recent report of NETs and LDGs from skin biopsies of patients with pyogenic arthritis, pyoderma gangrenosum and acne (PAPA) syndrome in association with increased tissue IL-1β, IL-8 and IL-17 (34). The serum from patients with PAPA syndrome is deficient in NETs clearance and was able to induce NETosis in healthy control neutrophils in an IL-1β dependent manner (34), similar to our findings.

Regarding the relationship between LDGs, NETs and the clinical features of AOSD, the spectrum of joint damage in AOSD can range from arthralgias to true erosive arthritis (2). The local damage and arthritis seen in these subclasses of disease involves proteases and reactive oxygen species, especially the expression of myeloperoxidase, metalloprotease 8 and 9 and IL-8 (13), which are components of the NETs and support our results as patients with arthritis show a trend towards higher LDGs and NETs.

Furthermore, most patients with AOSD present concomitant skin rash with acute episodes of arthritis and neutrophils seem to be key players in the pathogenesis of these manifestations since several molecules involved in their activation, such as IL-18 (33) have been proposed as predictors of chronic inflammatory diseases.

**Supplementary Table 1.** Clinical characteristics of patients with AOSD at diagnosis and at the time of the evaluation.

<table>
<thead>
<tr>
<th>Features</th>
<th>At diagnosis</th>
<th>Current</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>29 (96.7)</td>
<td>4 (13.3)</td>
</tr>
<tr>
<td>Dermatosis</td>
<td>25 (83.3)</td>
<td>4 (13.3)</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>24 (80)</td>
<td>10 (33.3)</td>
</tr>
<tr>
<td>Arthritis</td>
<td>21 (70)</td>
<td>7 (23.3)</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>15 (50)</td>
<td>2 (6.7)</td>
</tr>
<tr>
<td>Pharyngodynia</td>
<td>13 (43.3)</td>
<td>2 (6.7)</td>
</tr>
<tr>
<td>Myalgias</td>
<td>13 (43.3)</td>
<td>1 (3.3)</td>
</tr>
<tr>
<td>Serositis</td>
<td>4 (13.3)</td>
<td>2 (6.7)</td>
</tr>
<tr>
<td>Haematomegaly</td>
<td>4 (13.3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>3 (10)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Pneumopathy</td>
<td>3 (10)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>2 (6.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Complications</td>
<td>2 (6.7)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>
arthritis in AOSD (35). Neutrophils from patients with AOSD show an activated phenotype with expression of the high-affinity IgG receptor (CD64) which is involved in proliferation and diapedesis, correlates with disease activity (36) and is overexpressed in patients with JIA, composing an activation signature, with potential implications in joint damage (13). This supports our results and revisits the relevance of LDGs as a new subset of neutrophils that potentially promote the local insult at the synovial membrane in response to different types of molecules such as IL-18 and the expression of CD64, conditioning, at least partly, the clinical progression of the arthritis.

We propose that normal and low-density granulocytes may amplify and perpetuate the pathogenic auto-inflammatory response in AOSD. In autoimmune diseases such as SLE and after the stimuli with PMA and Granulocyte-colony stimulating factor (G-CSF), LDGs produce higher levels of TNF-α, IFN-γ and IL-6 in comparison to healthy control neutrophils and autologous normal density neutrophils (NDN) (12), which are key cytokines in the pathophysiology of AOSD.

Additionally, even when there is evidence that propose functionally different properties compared with normal density granulocytes (NDN) (27), in the majority of the pathogenic contexts, LDGs are characterised by an enhanced production of NETs (12-16), and in this study we found that patients with active AOSD have a higher amount of plasmatic DNA-NE complexes. The NETs are known to be an important source of DAMPs including histones and DNA (8). When DNA binds to absent in melanoma 2 (AIM2), it promotes the assembly of the inflammasome recruiting the associated speck-like protein containing CARD (ASC) and activating caspase 1 (37), which induce the liberation of IL-18, IL-1 and promotes pyroptosis (37). Accordingly, intra-articular injections of NE into knee joints of mice induced acute inflammation and pain in a proteinase-activated receptor-2 (PAR2)-dependent manner. The activation of this receptor and the phosphorylation of p44/42 MAPK pathway seem to be critical to the arthritis mediated by NE, however there is no experimental data that demonstrate the pathogenic role of DNA and NE together, forming the DNA-NE complex, or the tissue damage mediated by NETs release (38). These data are in agreement with our findings of higher NETosis in patients with arthritis. On the other hand, an uncontrolled inflammation and abnormal function of PRR such as TLR7 and TLR9 seems to be crucial in AOSD pathophysiology (19).

Interestingly, intra-articular injections of NE induce the liberation of IL-18, IL-1 and promotes pyroptosis (37). Accordingly, intra-articular injections of NE into knee joints of mice induced acute inflammation and pain in a proteinase-activated receptor-2 (PAR2)-dependent manner. The activation of this receptor and the phosphorylation of p44/42 MAPK pathway seem to be critical to the arthritis mediated by NE, however there is no experimental data that demonstrate the pathogenic role of DNA and NE together, forming the DNA-NE complex, or the tissue damage mediated by NETs release (38). These data are in agreement with our findings of higher NETosis in patients with arthritis. On the other hand, an uncontrolled inflammation and abnormal function of PRR such as TLR7 and TLR9 seems to be crucial in AOSD pathophysiology (19).

In fact, myeloid dendritic cells from patients with AOSD have higher expression of TLR7, which correlates with the disease activity score, promote the secretion of IFN-α and decreases after treatment (39) and imiquimod (a potent agonist of TLR7) can stimulate PBMCs from patients with AOSD and increase the mRNA expression of NLRP3 (7). TLR7 and TLR9 receptors are targets capable of detecting ssRNA and dsDNA from the NETs, respectively; and in this way promote the synthesis of IFN-α by priming more neutrophils with potential susceptibility to make spontaneous NETs (33), in agreement with our data of enhanced spontaneous NETosis in patients with AOSD. Therefore, in AOSD, NETs may be an important source of DAMPs, which are able to activate the inflammasome and TLR pathways in innate immune cells.

NETs may be a key connection between the excessive activation of neutrophils and macrophages because the former are able to attract monocytes to inflammation sites and alarmins from neutrophils such as HMGB1, S100A8 and S100A9 promote macrophage differentiation to a pro-inflammatory phenotype (40). After whole blood flow cytometry analysis, it was shown that patients with AOSD have a higher expression of CD11b and CD32. CD11b is a PRR that recognises β-glucan but also HMGB1 and the soluble form of RAGE (a decoy receptor for advanced glycation end products (RAGE) (43). Besides, HMGB1 has been related to SLE cutaneous manifestations (44) and previous studies had demonstrated higher serum levels of HMGB1 in patients with AOSD with pharyngodynia and cutaneous manifestations and correlated with the disease activity index and CRP levels (45). Similar to our results, higher amount of extracellular HMGB1 was found in biopsy of lesions from patients with cutaneous lupus and it has been suggested that keratinocytes, innate immune and necrotic cells release this alarmin (46). Our findings suggest that NETs are a source of extracellular HMGB1, which could promote skin damage in AOSD. Additionally, HMGB1 contained in NETs is able to induce macrophage pyroptosis through RAGE activation leading to the production of TNF-α and IL-1β in an animal model of sepsis (47). Therefore, the secretion of NETs containing HMGB1 in AOSD may promote the secretion of cytokines that produce fever (TNF-α and IL-1β) by macrophages and could induce dermatitis as shown in our study. Whilst HMGB1 is augmented, the soluble form of RAGE (a decoy receptor inhibiting the deleterious effect of HMGB1) has been found reduced in synovial fluid of patients with JIA and childhood SLE, correlating with early onset of disease (48), but this has not been studied in AOSD. Interestingly, we found that after incubation of healthy control neutrophils with sera from patients with active AOSD, there was an induction of NETosis that was not observed with healthy control sera. This may be ex-
plained by the fact that the two most relevant cytokines in AOSD are IL-1β and IL-18 (49). These cytokines are elevated during disease activity (50) and both are able to induce NETosis, especially, the stimulation with recombinant IL-18 leads to the release of NETs capable to induce activation of caspase-1, the key enzyme of the inflammasome that activates IL-1β and IL-18, which constitutes a positive feedback loop that enhances the extracellular trap formation, the synthesis of IL-1β and IL-18 and perpetuates the inflammatory milieu seen in these patients (51).

Finally, although this is a transversal study with a small sample size, it represents a thorough analysis of NETosis in AOSD and demonstrated an association between NETs and the clinical features of the disease.

Conclusion
In summary, patients with active AOSD have a higher percentage of LDGs and NETs and correlates with clinical manifestations. NETs are a potential source of extracellular HMGB1, which may be related with the induction of dermitis and fever in AOSD. The serum from patients with active AOSD is able to induce NETosis and this mechanism of cell death may be a key factor for the induction and perpetuation of inflammation in this disorder.

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