



Original Article

Neutrophils contribute to vasculitis by increased release of neutrophil extracellular traps in Behçet's disease



Rémi Safi^a, Romy Kallas^b, Tara Bardawil^c, Carl Joe Mehanna^d, Ossama Abbas^c, Rola Hamam^d, Imad Uthman^b, Abdul-Ghani Kibbi^c, Dany Nassar^{a,c,e,*}

^a Department of Anatomy, Cell Biology and Physiological Science, American University of Beirut, Beirut, Lebanon

^b Division of Rheumatology, Department of Internal Medicine, American University of Beirut Medical Center, Beirut, Lebanon

^c Department of Dermatology, American University of Beirut Medical Center, Beirut, Lebanon

^d Division of Ophthalmology, Department of Surgery, American University of Beirut Medical Center, Beirut, Lebanon

^e Hôpital Cochin Tarnier, Département de Dermatologie, Université Paris Descartes, Paris, France

ARTICLE INFO

Article history:

Received 28 March 2018

Received in revised form 14 August 2018

Accepted 28 August 2018

Keywords:

Neutrophils

Neutrophil extracellular traps

Behçet's disease

Vasculitis

ABSTRACT

Background and objectives: Behçet's disease (BD) is a multi-system inflammatory disorder that can cause vasculitis. Here we questioned whether Neutrophils in BD cause vasculitis *via* releasing Neutrophil Extracellular Traps (NETs), a process called NETosis.

Methods: Circulating neutrophils were isolated from a cohort of Middle Eastern BD patients with an active disease and healthy volunteers. The percentage of NETs release was monitored in neutrophils stimulated or not with BD serum, and treated or not with Colchicine, Dexamethasone, Cl-amidine or N-Acetyl Cysteine (NAC). The mRNA expression levels of PAD4 (a key enzyme in NETosis) was also assessed. The effect of NETs on the proliferation and cell death of endothelial cells was investigated using an *in vitro* co-culture model. The presence of NETs in skin tissues of BD patients was examined using immunolabeling of NETs associated proteins.

Results: Circulating Neutrophils from BD patients were more prone to release NETs *in vitro* and expressed higher levels of PAD4 compared to healthy volunteers. Spontaneous NETs formation in BD neutrophils was inhibited by Colchicine and Dexamethasone, two drugs used to treat BD. NETs formation was also inhibited by Cl-amidine, a specific PAD4 inhibitor, and by NAC, a ROS inhibitor. Interestingly, serum from BD patients stimulated circulating neutrophils from healthy volunteers to release more NETs and increased their mRNA PAD4 expression. Moreover, endothelial cells cultured in the presence of NETs from BD patients showed a decrease in proliferation and an increase in apoptosis and cell death. Finally, NETosis was predominantly identified around affected blood vessels in biopsies of vasculitis from BD patients.

Conclusion: Our results provide evidence on the implication of NETosis in the pathophysiology of BD especially in inducing vasculitis.

© 2018 Japanese Society for Investigative Dermatology. Published by Elsevier B.V. All rights reserved.

1. Introduction

Behçet's disease (BD) is an inflammatory multi-system disorder causing recurrent oral and genital ulcers, uveitis, and vasculitis, in addition to cutaneous, neurological, gastrointestinal,

articular and pulmonary manifestations [1]. Mild cases are treated with Colchicine, an anti-inflammatory drug. Severe cases can be life threatening and require systemic steroids, anti-TNF α drugs and immunosuppressants. Its pathophysiology is poorly known. Genetic studies identified several susceptibility loci implicating MHC1 and innate immunity genes namely HLA-B51, ERAP1, IL10, IL23R-IL12RB2, and IL1A-IL1B [2–4]. The inflammatory process in damaged organs of affected individuals is nonspecific but often exhibits an abundant neutrophil infiltrate, vasculitis and thrombosis [5]. Neutrophils from patients having severe BD retain higher oxidative burst activity and increased production of IL8 compared to patients with a mild BD [6]. Circulating neutrophils from BD patients produce increased levels of Reactive Oxygen Species (ROS) and show higher NADPH

Abbreviations: BD, Behçet's disease; NETs, neutrophil extracellular traps; PAD4, peptidylarginine deiminase 4; ROS, reactive oxygen species; PR-3, proteinase-3; MPO, myeloperoxidase; ANCA, anti-neutrophil cytoplasmic autoantibodies; SVV, small vessel vasculitis; His-3-cit, citrullinated histone 3; HAEC, human aortic endothelial cells; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate.

* Corresponding author at: Department of Dermatology, American University of Beirut, P.O. Box 11-0236, Riad El Solh, Beirut, Lebanon.

E-mail address: dn18@aub.edu.lb (D. Nassar).

<https://doi.org/10.1016/j.jderm.2018.08.010>

0923-1811/© 2018 Japanese Society for Investigative Dermatology. Published by Elsevier B.V. All rights reserved.

activity compared to healthy controls [7]. These two neutrophil properties significantly correlate with fibrinogen clotting ability, providing a link between neutrophil activation and thrombotic events in BD [7].

Neutrophils are essential effector cells for the innate immune system. In addition to phagocytosis and release of antimicrobial substances, neutrophils can trap and kill bacteria by extracellular extrusion of nuclear DNA forming Neutrophil Extracellular Traps (NETs) [8]. This unique cell death program is called NETosis. However, NETs can also occur in the absence of microbial infection and induce tissue damage. In diabetes, NETs contribute to wound healing impairment, a major source of morbidity and mortality in this disease. Neutrophils from diabetic patients are more prone to undergo NETosis than those from normoglycemic individuals, and inhibiting NETs formation accelerates skin wound healing in diabetic mice [9]. When stimulated by Neutrophil Cytoplasmic Autoantibodies (ANCA), neutrophils from Small Vessel Vasculitis (SVV) patients release NETs that contain Proteinase-3 (PR-3) and Myeloperoxidase (MPO), which are the targets of ANCA in this disease [10]. Further, NETs were shown to be deposited in kidney specimens from patients with SVV glomerulonephritis [10]. The implication of NETs in the pathophysiology of BD is still poorly investigated. Recently, circulating neutrophils from BD patients having an active disease were shown to release more NETs in response to circulating factors, namely CD40L, compared to neutrophils from inactive disease [11].

In this study, we hypothesized that neutrophils contribute to vasculitis in BD by releasing NETs. We provide additional evidence that neutrophils from active BD patients show increased NETosis and that NETs are present in biopsies of cutaneous vasculitis and panniculitis in BD patients. We also performed functional studies on the cytotoxic effect of NETs on endothelial cells.

2. Material and methods

2.1. Patients

Patients with active BD (n=31) consecutively visiting the American University of Beirut Medical Center during 2016–2018 were enrolled in the study (Table 1). Healthy volunteers matched for age and sex were selected as normal controls. Blood samples were collected from patients with or without treatment having an active state of the disease. BD was diagnosed according to the International Study Group criteria. The study protocol was in accordance with the Institutional Review Board of the American University of Beirut. Informed consent was obtained from patients and control subjects.

2.2. Neutrophils isolation

Peripheral blood samples were collected in EDTA tubes from BD patients and controls. Blood was layered on Histopaque 1119 (Sigma, Saint Louis, Missouri, USA) and centrifuged for 20 min at 800 × g. The plasma layer was collected and stored at –80 °C for further experiments, whereas the interphase and the erythrocytes layers were discarded. The granulocyte-rich layer was collected and washed in PBS (Sigma, Saint Louis, Missouri, USA) (without calcium and magnesium). The granulocytes were then layered on a discontinuous Percoll gradient (85%, 80%, 75%, 70%, 65%) (Sigma, Saint Louis, Missouri, USA) and centrifuged for 20 min at 800 × g. The fractions between 80% and 65% Percoll layers were collected and washed in PBS. Cells were counted and kept in medium for culture or in PBS for RNA extraction experiments (Supplementary Figure 1). The purity of

Table 1

Demographic and clinical characteristics of Behçet's disease patients.

Patient Characteristics		n (%)
Gender	Female	9 (29%)
	Male	22 (71%)
Age (years)	<20	1 (3%)
	20–30	9 (29%)
	30–40	8 (26%)
	40–50	9 (29%)
	>50	4 (13%)
Family history of BD	Yes	6 (19%)
	No	14 (45%)
	n/a	11 (36%)
Current treatment	Colchicine	13 (39%)
	Oral steroids	5 (16%)
	MTX	3 (10%)
	Azathioprine	3 (10%)
	Infliximab	2 (6%)
	Adalimumab	7 (22%)
	Others	8 (26%)
Behçet's Disease Criteria		n (%)
	Recurrent oral ulcers	27 (87%)
	Recurrent genital ulcers	13 (42%)
	Other skin manifestation	
Ocular manifestations	Panniculitis	6 (19%)
	Pseudofolliculitis	7 (23%)
	Vasculitis	1 (3%)
Rheumatological manifestations	Anterior uveitis,	18 (58%)
	posterior uveitis,	
	panuveitis, other	
Neurological manifestations	Arthritis	14 (45%)
	Yes	4 (13%)
Gastrointestinal manifestations	Yes	1 (3%)
	Thrombotic events	Yes

granulocytes was assessed by morphological examination of neutrophilic elastase (Abcam, #ab68672, Cambridge, United Kingdom) immunofluorescence labelling (Supplementary Figure 2). Using this staining, neutrophils enrichment was more 95%. The viability of the cells was more than 90% as determined by trypan blue exclusion test.

2.3. NETs visualization and quantification: stimulation and inhibition studies

Neutrophils were seeded into 96 well-plates (30 000 cells/well) in RPMI 1640 (Sigma, Saint Louis, Missouri, USA) supplemented with 10% FBS (Sigma, Saint Louis, Missouri, USA), 10 nM HEPES (Sigma, Saint Louis, Missouri, USA) and 2 mg/mL human serum albumin (Sigma, Saint Louis, Missouri, USA). The plates were incubated for 15 min at 37 °C to allow cells adhesion. The time kinetic of NETs release was assessed by fixation of unstimulated neutrophils after 1 h, 2 h, 3 h and 4 h in culture using 2% paraformaldehyde.

For the inhibition studies, neutrophils from BD patients were seeded into 96 well-plates (30 000 cells/well) and treated with either Colchicine (10 µg/mL) (Sigma, Saint Louis, Missouri, USA), Dexamethasone (5 µM) (Medochemie Limited, Limassol, Cyprus), Cl-amidine (10 µM) (Sigma, Saint Louis, Missouri, USA) or N-Acetyl Cysteine (NAC, 15 mM). For stimulation studies, neutrophils from healthy controls were seeded into 96 well plates (30 000 cells/well), stimulated or not with serum from BD patients (diluted 20%), and treated or not with Colchicine, Dexamethasone and Cl-amidine. All cultures were left for 2 h at 37 °C, then either fixed with 2% paraformaldehyde for labelling and counting; or washed in PBS and stored for further mRNA extraction.

After PBS washes, fixed cells were incubated with Hoechst 33342 (0.5 mg/mL) (Molecular probes, Eugene, Oregon, United States) for 10 min, followed by the addition of Sytox green (5 µM) (Invitrogen, Carlsbad, California, United States) for 30 min. NETs were visualized using fluorescence microscopy (Carl Zeiss,

Oberkochen, Germany). For NETs quantification, at least ten images were randomly taken from different regions of the wells (duplicate wells for each condition). The image files were loaded as separate image stacks in ImageJ software for cell count. The percent of NETs formation was calculated as follows: (Number of cells showing NETosis/Total number of cells) x 100.

2.4. PAD4 mRNA level analysis

RNA extraction was performed from isolated neutrophils using RNeasy Plus Mini kit (Qiagen, Hilden, Germany) as per manufacturer instructions. RNA concentration and purity were assessed using Nanodrop spectrophotometer (Denovix, Delaware, USA). cDNA synthesis was performed on 1 µg of extracted RNA using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, California, United States). RT-PCR was performed using specific primers for PAD4 gene in a CFX96 system (Bio-Rad, Hercules, California, United States). Gene expression levels were calculated relative to the 18 s housekeeping gene.

PAD4: F: ACCAGAGCTGTGAAAGATCAGA

R: TCACAGTTCACCAGCAGGAT

18 s: F: CAGCCACCCGAGATTGAGCA

R: TAGTAGCGACGGCGGTGTG

2.5. Immunofluorescence

Skin tissues: formalin fixed paraffin-embedded tissues from seven BD patients were collected in accordance with the Institutional Review Board of the American University of Beirut. Sections of 4 µm-thick tissues were mounted on glass slides. After deparaffinization in xylol, the slides were immersed in distilled water. Sections were pre-heated using heat-mediated antigen retrieval with sodium citrate buffer (for elastase staining) or Tris-EDTA buffer (for Citrunillated Histone 3, His-3-Cit staining) for 20 min.

Cells: neutrophils isolated from BD patients were cultured on glass coverslips for 4 h at 37 °C and fixed in 2% paraformaldehyde for 15 min.

On both skin tissues and neutrophils, blocking was performed for 1 h at room temperature with 3% normal goat serum (Nippon Chemi-Con, Japan). Two primary antibodies for Elastase (5 µg/mL, Abcam, #ab68672, Cambridge, United Kingdom) and His-3-Cit (10 µg/mL Abcam, #ab5103, Cambridge, United Kingdom) were used for the *in vivo* staining. In addition to these antibodies, anti-MPO (5 µg/mL, Abcam, #ab45977, Cambridge, United Kingdom) and anti-PR-3 (4 µg/mL Abcam, #ab150511, Cambridge, United Kingdom) were used for the *in vitro* staining. Tissues and cells were placed in a humidified chamber and incubated overnight at 4 °C with the corresponding primary antibodies. After PBS-tween washes, cells were next incubated with the secondary antibody Alexa Fluor 488 of goat anti-rabbit IgG antibody (1 µg/mL, Invitrogen, Carlsbad, California, United States). DNA was stained by addition of Hoechst 33342 (0.5 mg/mL, Molecular probes, Eugene, Oregon, United States) dye. After washing, coverslips were mounted on slides with an anti-fade reagent. Slides were examined using a laser scanning confocal microscopy, LSM710 (Carl Zeiss, Germany, Oberkochen, Germany).

2.6. Endothelial cells culture with NETs, cell cycle analysis and MTT proliferation assay

Unstimulated neutrophils were seeded into 6 well plates and incubated at 37 °C for NETs release. After 4 h of culture, neutrophils were removed using multiple PBS washes and Human Aortic Endothelial Cells (HAECs) were directly added to the wells containing NETs. The purity of NETs after PBS washes was assessed by counting remaining cells in the wells after Hoechst 33342 staining (Supplementary Figure 3). Wells containing HAECs alone

(without NETs) and NETs alone in Endothelial cell growth medium (Lonza, Basel, Switzerland) were used as controls. After 24 h of co-culture, cell cycle and proliferation of HAECs were assessed.

For the cell cycle analysis, supernatant and adherent HAECs were harvested and fixed using ethanol. Fixed cells were then washed with PBS, stained using Propidium Iodide (PI, 1 mg/mL) (Sigma, Saint Louis, Missouri, USA) for 15 min in the dark and the fluorescence was measured using flow cytometry (Guava, easycyte, Merck Millipore, Massachusetts, United States). A total of 10 000 gated events were acquired in order to assess the proportions of cells in different stages of the cell cycle. Analysis of cell cycle distribution was performed using the machine's software.

For the cell proliferation assay, 5 mg/ml of MTT reagent (Promega, USA) was added for 4 h, followed by the solubilization solution (100 µl/well). The optical density was measured after 24 h of incubation, at 570 nm by the Multiskan Ex spectrum. The percentage of surviving cells was determined by comparing the average absorbance of the co-cultured cells with the average of the control cells. Results are presented as percent of control.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 (San Diego, CA, USA). Samples statistics are reported as means ± SEM. Parametric tests were applied for two-group comparisons using unpaired t tests with two-tailed p values. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Neutrophils from BD patients are more prone to NETosis

Circulating neutrophils were isolated from the peripheral blood of BD patients (n = 31) and healthy donors matched for age and sex using a gradient centrifugation method (Supplementary Figure 1). Cells were incubated at 37 °C for their optimal growth and the spontaneous release of NETs was monitored after 1 h, 2 h, 3 h and 4 h of culture. The percentage of neutrophils undergoing NETosis *in vitro* was significantly higher in BD patients compared to controls at 1 h (11 ± 2% vs 4 ± 1%; p < 0.01), 2 h (22 ± 4% vs 7 ± 1%; p < 0.01), 3 h (22 ± 3% vs 7 ± 2%; p < 0.005) and 4 h (25 ± 3% vs 6 ± 1%; p < 0.005) (Fig. 1A and B). To further confirm that circulating neutrophils from BD patients are primed to NETosis, we assessed the mRNA expression levels of peptidylarginine deiminase 4 (PAD4) enzyme in neutrophils from BD and healthy individuals. PAD4 promotes citrunillation of arginine residues on chromatin, which is a key event in NETosis [12]. As shown in Fig. 1C, PAD4 mRNA levels were significantly higher with a 3 times fold (3.1 ± 0.6, p < 0.01) increase in BD patients compared to controls (1 ± 0.2).

To characterize the protein composition of NETs from BD patients, immunofluorescence of NET-bound proteins previously identified in other inflammatory diseases, namely elastase, His-3-Cit, MPO, and PR-3 was performed. Confocal images confirmed the presence of all four proteins in the NETs of unstimulated neutrophils from BD patients *in vitro* (Fig. 1D). Interestingly, the expression of Elastase and His-3-Cit, which are ubiquitous components of neutrophils and NETs, is invariably present in all NETs from BD neutrophils. However, the expression of MPO and PR-3, which are essential components of NETs in SVV patients, was only present in a small fraction of NETs from BD patients.

3.2. NETs release is inhibited by Colchicine, Dexamethasone, Cl-amidine or N-Acetyl Cysteine and stimulated by serum from BD patients

In order to assess whether the drugs used to treat BD, PAD4 inhibition or ROS inhibition have an effect on NETs release, we treated isolated circulating neutrophils from BD patients with Colchicine,

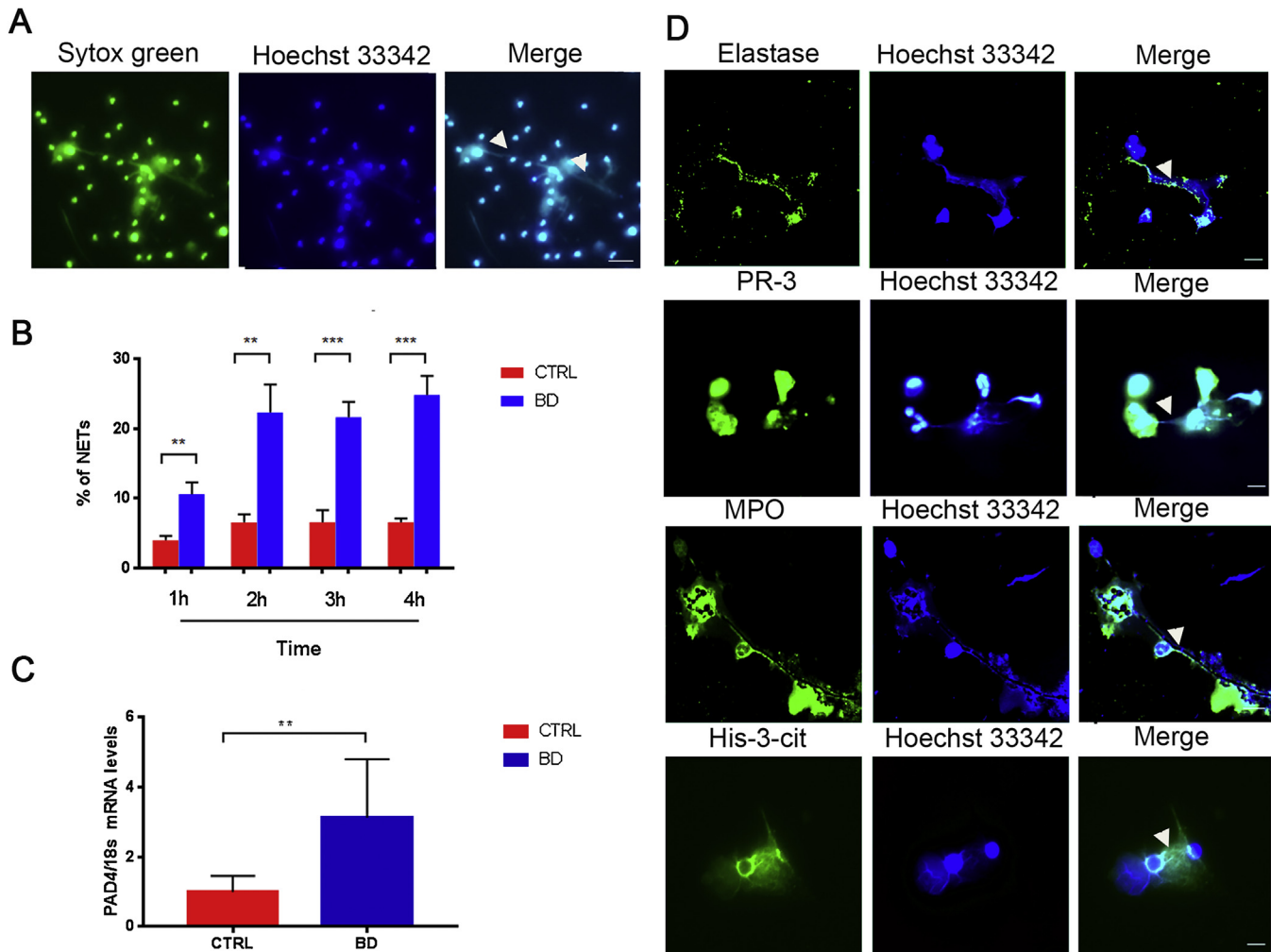


Fig. 1. A. NETs in unstimulated neutrophils from BD patients after 4 h in culture. Extracellular DNA was stained with Sytox green (left) and cellular DNA was stained with Hoechst 33342 (right). Arrows point to NETs forming neutrophils. Cells were observed under fluorescence microscopy. Scale bar, 50 μ m. B. Quantification of NETs in unstimulated neutrophils from BD patients (blue) (1 h n=5; 2 h n=6; 3 h n=5 and 4 h n=5) and healthy controls (red) (1 h n=5; 2 h n=6; 3 h n=5 and 4 h n=5). Mean percentage \pm SEM of NETs forming neutrophils is represented in the histograms with *t*-test **= $p < 0.01$, ***= $p < 0.001$. C. mRNA expression levels of PAD4 in circulating neutrophils from BD patients (n=8) and controls (n=7). Results are represented as fold expression normalized to the control with mean \pm SEM. 18 s was used as a housekeeping gene. *T*-test, **= $p < 0.01$. D. Immunostaining for Elastase, PR-3, MPO and His-3-cit in unstimulated neutrophils from BD patients *in vitro*. Cells were incubated for 4 h in culture. Cell nuclei were counterstained with Hoechst 33342 dye. Arrows point to NETs. Scale bar, 10 μ m.

Dexamethasone, Cl-amidine (a specific PAD4 inhibitor) or NAC (a ROS inhibitor). Interestingly, Colchicine, Dexamethasone, Cl-amidine and NAC significantly inhibited NETs release after 2 h in culture, respectively to $9.4 \pm 1.9\%$ ($p < 0.05$), $9.4 \pm 1\%$ ($p < 0.01$), $8.3 \pm 0.3\%$ ($p < 0.05$) and $6.3 \pm 1.4\%$ ($p < 0.05$) compared to untreated neutrophils ($22.3 \pm 3.9\%$) (Fig. 2A). These results suggest that the therapeutic effect of Colchicine and Dexamethasone in BD patients is potentially mediated by inhibiting NETosis, and that PAD4 activity and ROS are required for NETs release in BD neutrophils. Furthermore, we investigated whether circulating neutrophils are primed to NETosis by soluble factors in the bloodstream. For this purpose, we stimulated isolated circulating neutrophils from healthy individuals with serum from active BD patients and assessed their NETs release. When stimulated with BD serum, neutrophils from normal individuals released significantly more NETs ($21.7 \pm 1.8\%$, $p < 0.005$) than neutrophils stimulated with normal control serum ($6.5 \pm 0.8\%$) (Fig. 2B). The expression of PAD4 enzyme was significantly up-regulated in BD serum-treated control neutrophils to 2.1 ± 0.5 folds ($p < 0.05$) when compared to unstimulated control neutrophils (Fig. 2C). The increase in NETs release was significantly inhibited when BD serum was simultaneously added with Cl-amidine ($10 \pm 1.2\%$, $p < 0.01$), while

Colchicine and Dexamethasone treatment failed to reduce serum-induced NETs release. Importantly, while Colchicine and Dexamethasone decreased spontaneous NETosis in BD neutrophils (Fig. 2A), they both failed to inhibit NETs release when BD neutrophils were stimulated with BD serum (Fig. 2D). Interestingly, Cl-amidine was still able to inhibit serum-induced NETosis in BD neutrophils ($21.7 \pm 4.6\%$ vs $37.7 \pm 3.2\%$, $p < 0.05$) (Fig. 2D). These results suggest that circulating factors during BD flares –rather than intrinsic properties of BD neutrophils– stimulate NETs release *via* PAD4 upregulation and activity.

3.3. NETs inhibit endothelial cell proliferation and induce cell death

Vascular events in BD include thrombosis and vasculitis. Previous studies have shown that activated neutrophils in BD producing reactive oxygen species alter fibrinogen structure which is associated with increased thrombosis [7]. Nevertheless, the effect of BD neutrophils and more particularly NETs in inducing vasculitis was never investigated. To address this question, circulating neutrophils isolated from BD patients were cultured for 4 h to release their NETs, then washed away and HAECs were added on top of the adherent

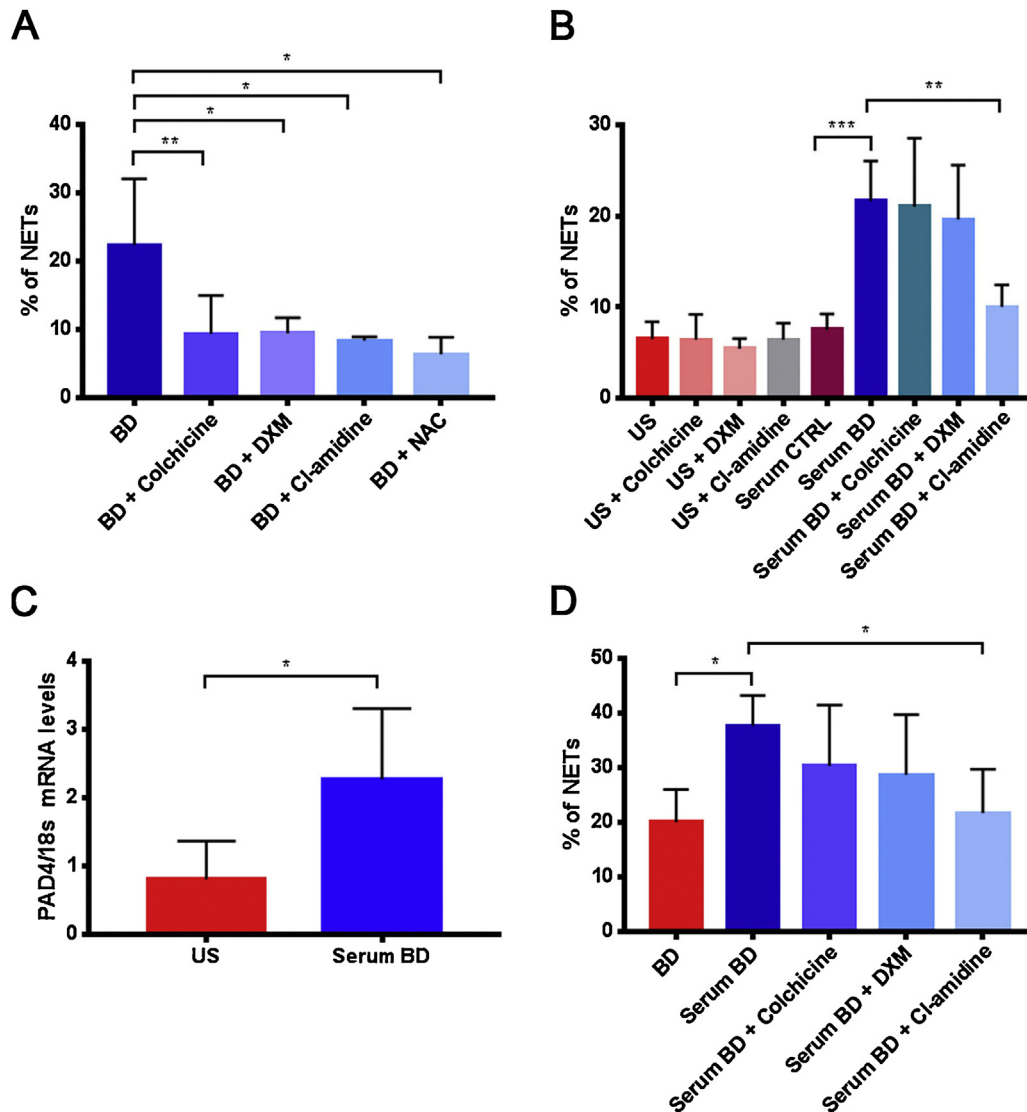


Fig. 2. A. Inhibition of NETs in BD neutrophils. Neutrophils from BD patients after 2 h in culture treated with Colchicine (10 μ g/mL, n = 8), Dexamethasone (DXM) (5 μ M, n = 5), Cl-amidine (10 μ M, n = 3) and NAC (15 mM, n = 3) represented as mean percentage of NETs \pm SEM, compared to non-treated BD neutrophils (n = 6). B, C and D. Stimulation of control or BD neutrophils with BD serum. (B) Unstimulated neutrophils treated with Colchicine (n = 5), DXM (n = 5), Cl-amidine (n = 5) and stimulated neutrophils with serum from BD patients (n = 6) and simultaneously treated with Colchicine (n = 5), DXM (n = 5), Cl-amidine (n = 4) and controls (n = 6), represented as mean percentage of NETs \pm SEM. (C) mRNA expression levels of PAD4 in neutrophils from healthy controls stimulated (n = 4) or unstimulated (US, n = 4) with serum from BD patients. 18 s was used as a housekeeping gene. (D) BD neutrophils stimulated with serum from BD patients (n = 3) and simultaneously treated with Colchicine (n = 3), DXM (n = 3) and Cl-amidine (n = 3). Results are represented as mean \pm SEM with *t*-test * = $p < 0.05$; ** = $p < 0.01$ and *** = $p < 0.005$.

NETs for 24 h. Following stimulation with NETs, cell cycle analysis was performed on HAECs using PI staining and flow cytometry. In the presence of NETs, a higher proportion of HAECs were accumulated in the pre G0/G1 cell cycle phase ($10.0 \pm 0.8\%$, $p < 0.01$), compared to $4.3 \pm 0.9\%$ in the absence of NETs (Fig. 3A and B). Accordingly, proliferation of HAECs is significantly reduced in the presence of NETs as assessed by an MTT assay (Fig. 3C). Taken together, these results provide evidence that NETs released by BD neutrophils cause an increase in endothelial cell death.

3.4. NETs are present in cutaneous vasculitis and panniculitis in BD

To investigate whether NETs are associated to vasculitis *ex vivo*, immunolabeling of Elastase and His-3-Cit proteins was performed on biopsies of various muco-cutaneous manifestations from seven BD patients (Table 2). Histone citrullination is a key event in chromatin decondensation and NETs formation and is used here as

a marker of NETs [12]. Although positively labelled cells for both markers were present in all screened tissues, we observed differences in the distribution of these proteins among different skin lesions. Neutrophils labelled with Elastase were diffusely present in lesions of vasculitis and panniculitis. Interestingly, Neutrophils that had undergone NETs labelled with His-3-Cit were focally present in proximity to vessels in lesions of vasculitis and in subcutaneous septa in lesions of panniculitis (Fig. 4 and Supplementary Figure 4). These results highlight the role of NETs in endothelial and tissue damage in BD.

4. Discussion

Our results show that BD neutrophils are stimulated by circulating factors to release NETs. We also provide evidence that Colchicine and Dexamethasone, two drugs used to treat BD, inhibit spontaneous NETosis in BD neutrophils and that ROS and

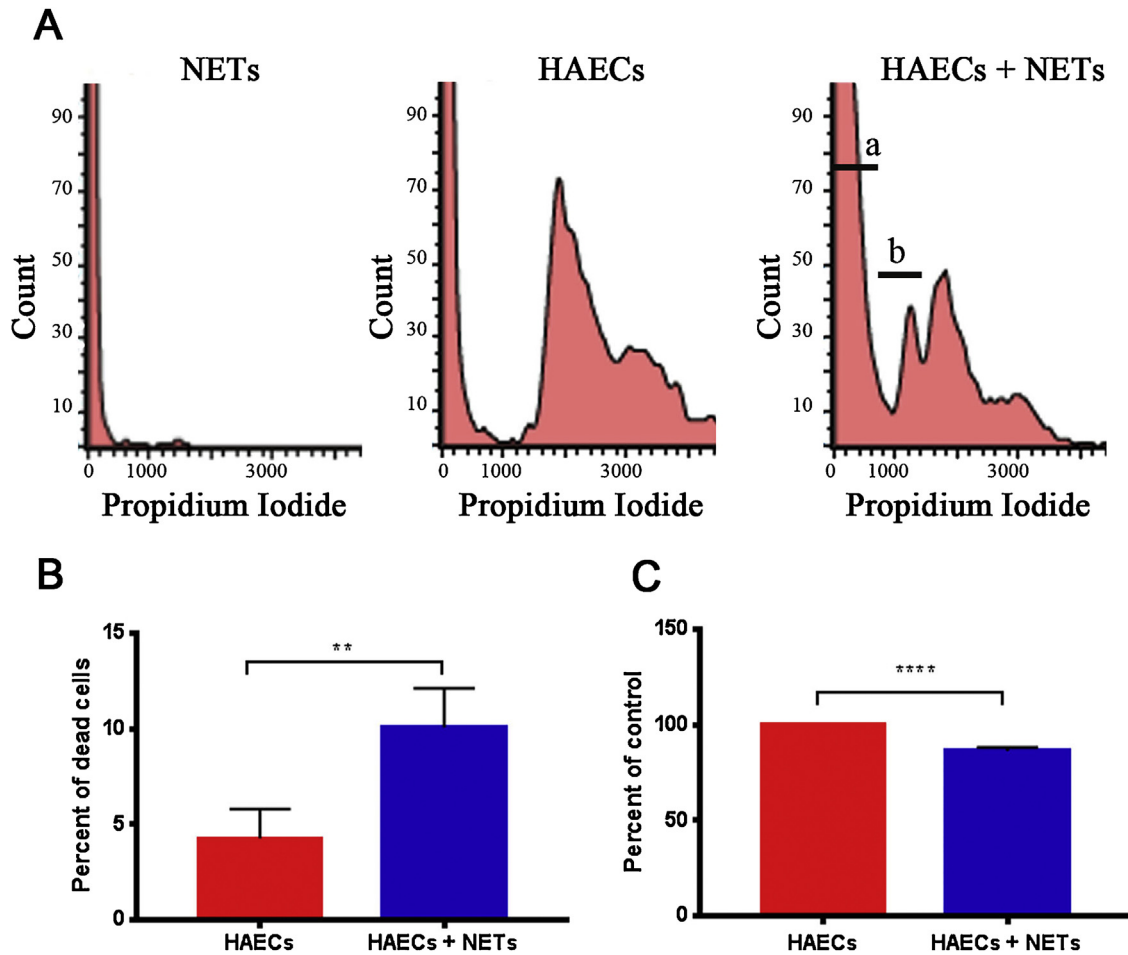


Fig. 3. Cell cycle analysis and cellular proliferation of HAECs cultured in the presence of NETs from BD neutrophils. **A.** Representative flow cytometry histograms showing the distribution of cell cycle phases after propidium iodide staining. (a): Debris and (b): apoptotic cells. NETs alone and HAECs alone were considered as controls. **B.** Quantification of the dead cell population (a + b) in HAECs alone and in the presence of NETs (n=6). **C.** Cellular proliferation of HAECs assessed by MTT assay (n=4). Data is presented as mean ± SEM with t-test **= p < 0.01 and ***= p < 0.005.

PAD4 are essential for NETs release. More interestingly, we demonstrate that NETs from BD neutrophils contribute to endothelial cell death and are present in skin lesions of vasculitis and panniculitis.

Few studies have addressed the role of NETosis in BD. In line with our results, a recent study by Perazzo *et al* showed that circulating neutrophils from active BD release more NETs than those from inactive BD and healthy controls [11]. In that study, the

authors performed a multiplex screening of cytokines and soluble receptors and found a number of soluble factors increased in the serum of active BD patients compared to healthy controls. The most elevated factor in their screening was CD40L. A number of other soluble factors were also reported, namely TNFα and Interferon γ, which are both potential NETs inducers in BD [11]. Interestingly, IL8 a known chemo-attractant of neutrophils, was not significantly increased in the serum of active BD [11]. This

Table 2
Neutrophils and NETs labelled with Elastase and Citrinillated Histone 3 in skin biopsies of various skin lesions from BD patients.

Patient	Clinical manifestation	Histopathological features	Staining	
			Elastase	His-3-Cit
1	Panniculitis	Septal panniculitis, perivascular neutrophilic infiltrate, mild/focal vasculitis	Diffuse, septal, perivascular	Focal, septal
2	Acute neutrophilic dermatosis	Diffuse dense neutrophilic infiltrate, necrosis of epidermis, mild/focal vasculitis	Diffuse, dermal	Focal, perivascular
3	Vasculitis	Septal panniculitis, vasculitis with fibrinoid necrosis	Diffuse, septal	Focal, septal
4	Panniculitis	Lobular panniculitis, dermal neutrophilic infiltrate, mild/focal vasculitis	Diffuse, dermal, septal, perivascular	Focal, perivascular
5	Panniculitis	Mild septal panniculitis, focal epidermal ulceration, mild/focal vasculitis	Diffuse, Septal, perivascular	Focal, septal, perivascular
6	Oral Ulcer	Epithelial necrosis with ulceration, dense lymphoneutrophilic infiltrate, no signs of vasculitis	Rare, scattered	Rare, scattered
7 A	Panniculitis	Septal panniculitis, septal lymphoneutrophilic infiltrate, perivascular inflammation,	Diffuse, dermal	Focal, septal
7 B	Genital Ulcer	Hyperplastic epidermis with focal ulceration, dense perivascular and interstitial lymphoneutrophilic infiltrate	Rare, scattered	Focal, Septal, perivascular

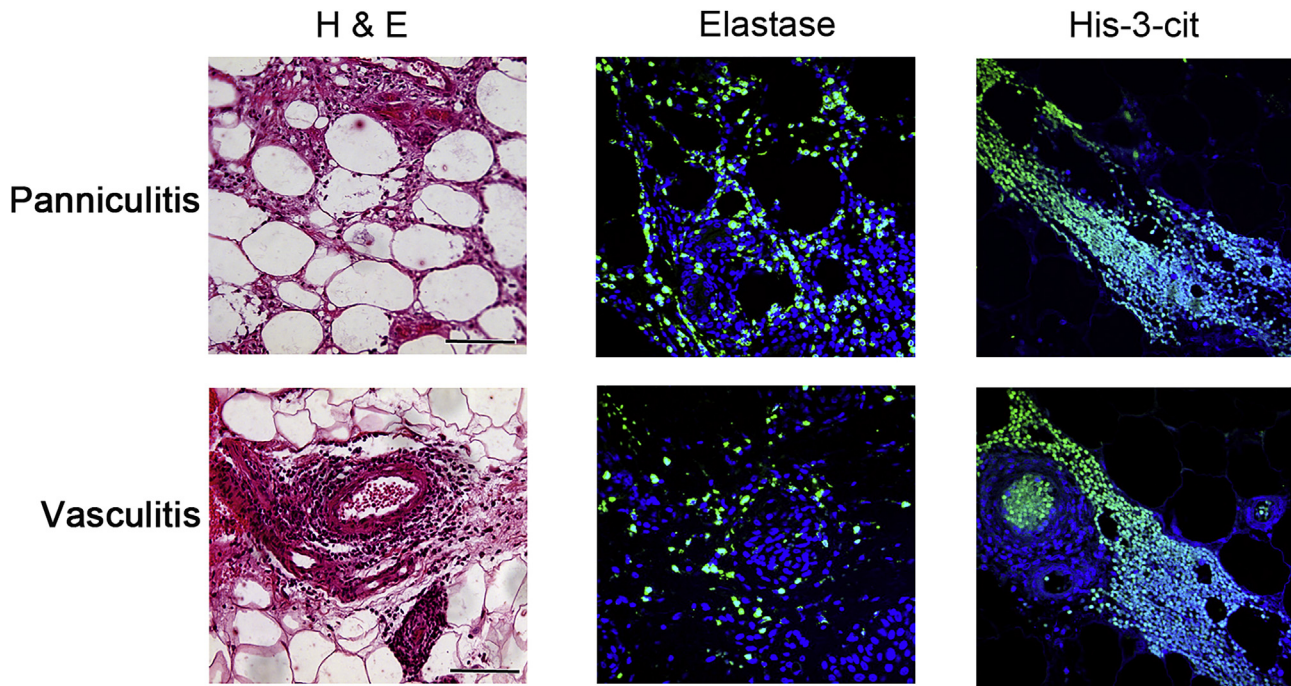


Fig. 4. Staining of BD vasculitis and panniculitis patients. Right panel: hematoxylin and eosin staining, left panel: immunostaining of Elastase and Citrullinated Histone 3 merged with Hoechst 33342 nuclear stain. Scale bar, 100 μm .

points towards distinct cytokines acting in attracting neutrophils and inducing NETosis. In addition to cytokines, auto-antibodies and ROS were shown to be inducers of NETosis in autoimmune diseases. In SVV, NETs are stimulated by ANCA autoantibodies and contain MPO and PR-3 which are the targets of auto-antibodies in this group of autoimmune diseases [10]. In lupus, the release of NETs is stimulated by disease specific immune complexes and is further amplified by mitochondrial ROS [13]. NETs in lupus contain oxidized mitochondrial DNA which stimulates type I Interferon signaling, a signature event in the disease pathophysiology [13]. In parallel to intrinsic factors, the role of saliva in stimulating NETosis was investigated in an attempt to provide a pathophysiological mechanism for the recurrent oral ulcers observed in BD [14]. Curiously, saliva from BD patients failed to induce NETs in neutrophils in contrast to saliva from healthy volunteers [14]. In our histology screening, we only had one biopsy of an oral ulcer and one of a genital ulcer. Those were the only biopsies that showed very rare NETs compared to biopsies of vasculitis and panniculitis lesions. Altogether, this suggests that NETosis in BD is not triggered by external factors but rather by circulating factors and is involved in specific -not all- tissue lesions.

Apart from histones and DNA, proteins bound to NETs participate to specific immune and inflammatory mechanisms in autoimmune and auto-inflammatory diseases. Proteins bound to NETs comprise proteases of neutrophilic granules such as elastase, MPO and PR-3. In our study, NETs from BD neutrophils invariably contained elastase, and to a lesser proportion MPO and PR-3. This variability is interesting and could attest of the different molecular mechanisms of NETs formations in response to different NETs inducers. In a recent study, *Kenny et al.*, showed that MPO, ROS and Protein Kinase C are differentially required for NETs release in response to different chemical and infectious inducers like PMA, *Candida albicans* and Group B *Streptococcus* [15]. Besides, the proteolytic activity of NETs induced by various stimuli is variable, which could reflect a variability in their protease contents [15].

The implication of NETosis in tissue damage was investigated in a number of autoimmune and auto-inflammatory diseases. In Familial Mediterranean Fever, a monogenic auto-inflammatory disease characterized by acute inflammatory attacks, NETs are released during disease attacks and are decorated with IL-1 β , a key cytokine in this disease [16]. Besides inflammation, NETs were shown to constitute scaffolds for thrombosis, and to stimulate aggregation of platelet and thrombus formation [17]. Thus, the increased NETosis in circulating BD neutrophils that we show here may explain the high susceptibility risk to thrombotic events that is observed in BD.

In kidney biopsies of SVV glomerulonephritis, NETs are present in proximity to affected glomeruli and are more abundant in specimens with dense neutrophil infiltrate, suggesting a role of NETosis in the active stage of vasculitis. In our series, NETs were present in lesions of vasculitis and panniculitis, both characterized by cell toxicity and necrosis. Moreover, HAECs undergo cell cycle arrest and cell death when cultured with NETs from BD patients. Neutrophils are known to induce endothelial cell death in lupus and psoriasis by secreting IL17 through NETosis [18,19]. In parallel, activated endothelial cells produce cytokines such as IL8 enabling transmigration of neutrophils [20]. Similar activation loops are likely to occur in BD and could explain the recurrent and multisystem vasculitis observed in this disease. Using other protocols, *Saffarzadeh et al* previously showed that NETs released after stimulation of healthy neutrophils with PMA, induce cell death *in vitro* when added on epithelial and endothelial cell lines in culture [21]. Interestingly, they showed that the NET-induced cytotoxicity is independent of their DNA content but is rather caused by histones. Taking together, these data provide strong evidence towards the implication of NETs in contributing to endothelial cytotoxicity and vasculitis in BD.

Both Colchicine and Dexamethasone inhibited spontaneous NETosis in BD neutrophils. Despite being an old drug, the mechanism of action of Colchicine in various inflammatory

conditions is not fully known. Its inhibitory effect on NETosis could be due to the inhibition of tubulin polymerization that is required for microtubule formation and degranulation of neutrophils [22]. Inhibition of degranulation might alter the function of proteases that are required for NETs release [15]. On the other hand, our data show that ROS inhibition by NAC inhibits NETosis in BD neutrophils. Since Dexamethasone inhibits ROS generation by neutrophils [23], that could mediate its inhibitory effect on NETosis. Nevertheless, neither Colchicine nor Dexamethasone were able to inhibit NETosis in control or BD neutrophils when stimulated with BD serum and only Cl-amidine, a specific PAD4 inhibitor successfully reduced NETosis in stimulated neutrophils. This could be explained by the need of a specific and potent inhibitor of NETosis, such as Cl-amidine, when neutrophils are continuously stimulated by internal factors.

Altogether, our findings provide evidence for the contribution of NETs in endothelial cytotoxicity and vasculitis in BD. Inhibiting NETosis might constitute an interesting therapeutic target in this disease and other autoimmune and auto-inflammatory disorders.

Author contributions

R.S. performed the experiments, analyzed the results and assisted in writing the manuscript; R.K. recruited patients and performed clinical data analysis; T.B., C.J.M., R.H., I.U., A.G.K. recruited patients; O.S. performed histopathological analysis; D. N. conceptualized and supervised the project, analyzed results and wrote the manuscript.

Funding source

This work has been supported by the MPP/URB grant [MPP 320141/URB 23272] from the American University of Beirut.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

The authors thank Dr. Abdul Fattah Al-Masri and Dr. Julien Torbey (American University of Beirut-Medical center) for recruiting BD patients, Dr. Marwan El-Sabban (American University of Beirut) for providing HAECs and for his scientific advice and technical help and Dr. Firas Kreidieh for his help in analyzing clinical data. We are grateful for the assistance of the IRB (ID: Der. DN.02) of the American University of Beirut. This work has been supported by the MPP/URB grant [MPP 320141/URB 23272] from the American University of Beirut.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jdermsci.2018.08.010>.

References

- [1] H. Yazici, et al., Behcet syndrome: a contemporary view, *Nat. Rev. Rheumatol.* (2018).
- [2] Y. Kirino, et al., Genome-wide association analysis identifies new susceptibility loci for Behcet's disease and epistasis between HLA-B*51 and ERAP1, *Nat. Genet.* 45 (2) (2013) 202–207.
- [3] E.F. Remmers, et al., Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behcet's disease, *Nat. Genet.* 42 (8) (2010) 698–702.
- [4] M. Takeuchi, et al., Dense genotyping of immune-related loci implicates host responses to microbial exposure in Behcet's disease susceptibility, *Nat. Genet.* 49 (3) (2017) 438–443.
- [5] M. Balabanova, et al., A study of the cutaneous manifestations of Behcet's disease in patients from the United States, *J. Am. Acad. Dermatol.* 41 (4) (1999) 540–545.
- [6] S.F. Perazzo, et al., Behcet's disease heterogeneity: cytokine production and oxidative burst of phagocytes are altered in patients with severe manifestations, *Clin. Exp. Rheumatol.* 33 (6 Suppl 94) (2015) S85–95.
- [7] M. Becatti, et al., Neutrophil Activation Promotes Fibrinogen Oxidation and Thrombus Formation in Behcet Disease, *Circulation* 133 (3) (2016) 302–311.
- [8] V. Papayannopoulos, Neutrophil extracellular traps in immunity and disease, *Nat. Rev. Immunol.* (2017).
- [9] S.L. Wong, et al., Diabetes primes neutrophils to undergo NETosis, which impairs wound healing, *Nat. Med.* 21 (7) (2015) 815–819.
- [10] K. Kessenbrock, et al., Netting neutrophils in autoimmune small-vessel vasculitis, *Nat. Med.* 15 (6) (2009) 623–625.
- [11] S.F. Perazzo, et al., Soluble CD40L is associated with increased oxidative burst and neutrophil extracellular trap release in Behcet's disease, *Arthritis Res. Ther.* 19 (1) (2017) 235.
- [12] Y. Wang, et al., Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation, *J. Cell Biol.* 184 (2) (2009) 205–213.
- [13] C. Lood, et al., Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease, *Nat. Med.* 22 (2) (2016) 146–153.
- [14] T. Mohanty, et al., A novel mechanism for NETosis provides antimicrobial defense at the oral mucosa, *Blood* 126 (18) (2015) 2128–2137.
- [15] E.F. Kenny, et al., Diverse stimuli engage different neutrophil extracellular trap pathways, *Elife* 6 (2017).
- [16] E. Apostolidou, et al., Neutrophil extracellular traps regulate IL-1beta-mediated inflammation in familial Mediterranean fever, *Ann. Rheum. Dis.* 75 (1) (2016) 269–277.
- [17] T.A. Fuchs, et al., Extracellular DNA traps promote thrombosis, *Proc. Natl. Acad. Sci. U. S. A.* 107 (36) (2010) 15880–15885.
- [18] E. Villanueva, et al., Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus, *J. Immunol.* 187 (1) (2011) 538–552.
- [19] A.M. Lin, et al., Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis, *J. Immunol.* 187 (1) (2011) 490–500.
- [20] A.K. Gupta, et al., Activated endothelial cells induce neutrophil extracellular traps and are susceptible to NETosis-mediated cell death, *FEBS Lett.* 584 (14) (2010) 3193–3197.
- [21] M. Saffarzadeh, et al., Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones, *PLoS ONE* 7 (2) (2012) e32366.
- [22] F. Roubille, et al., Colchicine: an old wine in a new bottle? *Antiinflamm. Antiallergy Agents Med. Chem.* 12 (1) (2013) 14–23.
- [23] P. Dandona, et al., Effect of dexamethasone on reactive oxygen species generation by leukocytes and plasma interleukin-10 concentrations: a pharmacodynamic study, *Clin. Pharmacol. Ther.* 66 (1) (1999) 58–65.