Original Article

Cytokine Network & NETs

(neutrophil extracellular traps / neutrophils / interleukin)

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Abstract: The process of forming and releasing neutrophil extracellular traps (NETs) can be regulated by exogenous and endogenous factors, including cytokines. The study aimed to assess the impact of pro-inflammatory cytokines, IL-15, IL-17, IL-18, and anti-inflammatory IL-10 on the formation of NETs, all in comparison to IL-8 and pathogenic factors: LPS, fMLP. Also, the expression of myeloperoxidase (MPO), one of the main elements of neutrophil traps, was evaluated. After isolating the neutrophils with Polymorphprep™, the cells were sorted using CD16 MACS® microbeads and incubated with selected factors. The formation of NETs was registered using a BD Pathway 855 microscope system and the expression of MPO was evaluated using flow cytometry. The amounts of circulating DNA in cell supernatants was fluorescently quantified. Microscopic photographs indicated that rhIL-15, rhIL-17, rhIL-18 and fMLP induce formation and release of NETs at a similar timespan, while in the presence of rhIL-10, the formation of the traps was delayed. The presence of the studied cytokines indicated two populations of neutrophils displaying differing MPO expression (MPO*low* and MPO*high*). Moreover, stimulation of neutrophils with LPS and fMLP revealed two populations of these cells that differed not only in the expression of MPO, but also in size.

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Abbreviations: ANA – antinuclear antibody, ANCA – anti-neutrophil cytoplasmic antibodies, CD – cluster of differentiation, fMLP – n-formyl-methionine-leucine-phenylalanine, FSC – forward scatter, GM-CSF – granulocyte-macrophage colony-stimulating factor, H – histone, HLA-DR – MHC class II cell surface receptor encoded by the human leukocyte antigen complex, IFN – interferon, IL – interleukin, LDGs – low density granulocytes, LPS – lipopolysaccharide, MPO – myeloperoxidase, NETs – neutrophil extracellular traps, PMNs – polymorphonuclear cells, rhIL – recombinant human interleukin, SSC – side scatter, TNF-α – tumour necrosis factor α.

Introduction

Neutrophils represent the first line of defence to bacterial factors. As specialized phagocytic cells, they migrate from the bloodstream to the affected tissue, where they successfully identify, bind, and devour pathogens. Phagocytized microorganisms are killed with the involvement of proteolytic enzymes, antibacterial proteins, and reactive oxygen species (Mantovani et al., 2011).

In 2004, Brinkmann et al. (2004) described an antibacterial action of neutrophils, unknown at the time, which involved creation of neutrophil extracellular traps (NETs).

Activated neutrophils experience a range of intracellular modifications that lead to the formation of NETs. In this process, the nuclei of neutrophils lose their form, chromatin becomes decondensed, the membranes surrounding the nucleus and granules dissolve, and lysosomal proteins bind with DNA strands. Such networks are then released outside the cell upon its altruistic death (Brinkmann et al., 2004; Fuchs et al., 2007).

Electron microscope images showed that NETs contain smooth, flexible DNA fibres with a diameter of 15–17 nm, and globular domains of approximately 25 nm, which can aggregate into larger structures, up to 50 nm in diameter. The networks were shown to contain numerous primary granule proteins (myeloperoxidase, neutrophil elastase, cathepsin G), secondary granule proteins (lactoferrin), tertiary granule proteins (gelatinase), and histones: H1, H2A, H2B, H3, H4 and H2A-H2B-DNA complex (Brinkmann et al., 2004).

The first reports on NETs indicated that they play a positive role in *in vivo* infections. The new neutrophil antibacterial mechanism confirmed their importance in

identifying and killing microorganisms. Studies have shown that the networks-traps retain their killing capacity even after the phagocytic mechanism in neutrophils is blocked. The ability to kill pathogens is exhibited not only by lysosomal cells and reactive oxygen species, but also by histone proteins present in the NETs (Brinkmann et al., 2004; Lee and Grinstein, 2004).

Extracellular neutrophil traps seem to be a form of innate immunological response that binds microorganisms and prevents their spread, providing at the same time high local concentration of antibacterial factors. Experimental studies have shown that NETs bind gram-positive and gram-negative bacteria, fungi, and protozoa. The formation of NETs has been shown in the course of appendicitis, necrotizing fasciitis, pre-eclampsia, dysentery, and pneumococcal pneumonia. Numerous NET structures are also found during infections with *Staphylococcus aureus*, *Salmonella*, *Shigella*, and also *Candida albicans* or *Leishmania amazonensis,* indicating that NETs are involved in killing a wide range of pathogens (Brinkmann et al., 2004; Lee and Grinstein, 2004; Guimarães-Costa et al., 2009; Yost et al., 2009).

There are individual reports about the unfavourable role of NETs in the human body. It has been indicated that in result of prolonged inflammation, accompanying NETs may induce autoimmunological processes (Guimarães-Costa et al., 2009; Fuchs et al., 2010; Hakkim et al., 2010).

Several factors regulating the formation of NETs have been identified, but the mechanism of forming and releasing the traps remains unexplained. The formation of NETs may be influenced by cytokines present in the cell environment, which affect all stages of the cell life. Other authors observed that pro-inflammatory cytokines induce formation of the neutrophil traps. Interleukin (IL)-8 or tumour necrosis factor α (TNF-α) have been shown to induce the formation of NETs (Brinkmann et al., 2004). This study aimed to assess the impact of other pro-inflammatory cytokines, i.e., IL-15, IL-17, IL-18, as well as anti-inflammatory IL-10, on the formation of neutrophil extracellular traps.

It has been shown that NETs are released when cells respond to LPS, and that is why it was decided to assess the impact of fMLP on the formation of extracellular traps (Brinkmann et al., 2004; Fuchs et al., 2007).

Material and Methods

Participants

The study involved a group of 15 healthy persons, volunteer blood donors. The clinical data on the healthy persons is presented in Table 1. (Approved by the Bioethics Committee of the Medical University of Bialystok No. R-I-002/574/2013)

Cell isolation

Upon the donor's consent, blood samples were extracted from the basilic vein and preserved with EDTA for cell isolation. After initial isolation with Polymorphprep™ (Axis-Shield PoC AS, Oslo, Norway), the cells were sorted using a magnetic MACS® Separator with CD16 Microbeads (for polymorphonuclear cells (PMNs), Miltenyi Biotec, Bergish Gladbach, Germany).

Cell incubation

Polymorphonuclear cells were suspended in a culture medium containing RPMI Medium 1640 (Gibco®, Life Technologies™), < 5% calf serum FBS Good (PAN Biotech, Aidenbach, Germany), antibiotics penicillin-streptomycin (Sigma Life Science, Darmstadt, Germany) in an amount of 10^5 per well for NET visualization and 5×10^5 for the flow cytometry method.

Then, the cells were incubated for 1 h in microplates (Microtest III-Falcon, BD Biosciences, Bedford, MA) at 37 °C, in an incubator with a flow of 5% CO_2 (NUAIRE™ US AUTOFLOW CO_2 Water-Jacketed Incubator, Plymouth, MN).

Neutrophils were incubated in the presence of rhIL-8 and rhIL-15 at a concentration of 100 ng/ml; rhIL-10, rhIL-17, rhIL-18 – 50 ng/ml; LPS – 10μg/ml; fMLP – 40 ng/ml.

Cell purity assessment

The purity of the collected cell suspensions was assessed in the "thick drop" preparations, using May-Grünwald-Giemsa staining (Aqua-Med, Lodz, Poland). The purity of all cell suspensions was 99.7 %.

Cell viability assessment

The viability of the cells was assessed immediately after separation in a light microscope using trypan blue (Lachema, Neratovice, Czech Republic). Following culture, the viability of PMNs after isolation was 98 % and after 1 h incubation, 97 %.

BD Pathway 855 microscopic system

The incubation of stimulated cells at 37 °C, in an incubator with a flow of 5% CO_2, was recorded in the incubation chamber with a BD Pathway 855 microscope system (San Jose, CA). The analysis involved NET structures: DNA (Hoechst 33342, Invitrogen™, Thermo Fisher Scientific, Waltham, MA) and myeloperoxidase (clone 8E6, Molecular Probes®, Life Technologies™, Darmstadt, Germany).

Table 1. Clinical data on healthy persons

			Blood smear [%]		
Healthy persons	**Age [years]**	**WBC [×10³ cells/μl]**	Band neutrophils	Polymorphonuclear cells	Mononuclear cells
N = 15	20–35	3.9–9.5	0–2	45–81	16–60

Flow cytometry

Following 1 h incubation, neutrophils were permeabilized using FACS Permeabilizing Solution 2 (BD Biosciences) and stained with anti-human myeloperoxidase monoclonal antibodies (clone 8E6, Molecular Probes®, Life Technologies™). Flow cytometric data were acquired in a FACSCalibur flow cytometer (BD Biosciences) and analysed with the use of FlowJo software (Tristar Inc., Ashland, OR). Neutrophils were gated on the basis of forward scatter (FSC) and side scatter (SSC) properties, and the frequency of myeloperoxidase-positive cells was presented as frequency of total neutrophils.

Circulating DNA quantification kit

The amounts of circulating DNA in cell supernatants were determined by an Abcam Circulating DNA Quantification Kit (Cambridge, UK) according to the instructions enclosed. DNA was fluorescently quantified.

Statistical analysis

Statistical analysis used the Student's *t*-test for paired samples (the results are presented as mean values) to indicate the differential tendencies in response to stimulation with the respective factors.

Results

Visualization of NETs

Microscopic photographs indicated that the pro-inflammatory cytokines, rhIL-15, rhIL-17, and rhIL-18, induce formation and release of NETs. The amount of traps created by neutrophils stimulated with rhIL-15, rhIL-17, and rhIL-18 is comparable with the amount of NETs generated by the cells in response to rhIL-8, whose involvement in the formation of NETs has already been proved. Significant amounts of NETs were observed upon 60 min incubation of neutrophils stimulated by rhIL-15, rhIL-17, and rhIL-18 compared to unstimulated cells. The networks released in response to stimulation with rhIL-15 rhIL-17 were visually similar to clouds and took up several times more space than the cells from which they originated. However, NETs induced by rhIL-18 were characterized by long fibres and strands (Fig. 1).

The experiments indicated that in the presence of anti-inflammatory rhIL-10, neutrophils showed a delay in formation of the traps in comparison to cells stimulated by pro-inflammatory cytokines. After 60 min incubation of neutrophils with rhIL-10, no NETs were found. Only after 120 min, a significant increase in the amount of traps was detected compared to unstimulated cells. However, the amount of NETs in the presence of rhIL-10 at the time was lower than in the cells incubated with rhIL-15, rhIL-17, and rhIL-18. Moreover, the microscopic images of the networks produced by neutrophils stimulated with rhIL-10 display different shapes than the networks created in the case of pro-inflammatory cytokines. The former traps seem more clustered around the cells that released them (Fig. 1).

Differences were also observed in the formation of neutrophil traps in response to rhIL-8 and LPS. The tests showed larger amounts of NETs formed in the course of neutrophil incubation with LPS than with rhIL-8, and with rhIL-15, rhIL-17 or rhIL-18. The spatial range of the traps from neutrophils stimulated with LPS was smaller than in response to rhIL-8 (Fig. 2). Tests with a strong neutrophil activator, fMLP, showed that it generated slightly less formation and release of NETs compared to LPS, but more in comparison to all of the studied cytokines (Fig. 1).

Table 2 summarizes the results of evaluation of the percentage of neutrophils forming NETs after 60 min stimulation.

Cytometric analysis of the myeloperoxidase content of neutrophils

Evaluation of the neutrophil MPO content with flow cytometry showed a significant decrease of MPO-positive neutrophils stimulated with rhIL-8 (14.8 %, $P = 0.0473$), rhIL-17 (27.72 %, $P = 0.0173$), and rhIL-18 (22.3 %, $P = 0.0451$) in comparison to non-stimulated cells (29.15 %). In the case of rhIL-15 stimulation, only a tendency to decrease the fraction of MPO-positive neutrophils was observed (26.5 %, $P = 0.0687$) (Fig. 3).

The results indicated the presence of two populations of neutrophils displaying differing MPO expression: cells with low MPO expression (MPO*low*) and cells with high MPO expression (MPO*high*) (Fig. 4). It was determined that in the case of rhIL-8 stimulation, the fraction of MPO*high* neutrophils is significantly decreased (4.99 %, $P = 0.0203$) in comparison to non-stimulated cells (14.31 %). After stimulation of neutrophils with rhIL-17, only a slight tendency to decrease the MPO*high* neutrophil fraction was observed (13.76 %, $P = 0.0605$). However, incubation of cells with rhIL-18 was associated with a significant decrease of MPO*low* neutrophils (11.5 %, $P = 0.0478$) in comparison to control cells (13.95 %) and showed a tendency to decrease the subpopulation of MPO*high* neutrophils (9.77 %, $P = 0.0513$) (Fig. 4a, 4b).

In the course of the study, it was shown that the stimulation of neutrophils with LPS, but also fMLP, leads to separation of these cells into two populations that significantly differ in size. Analysing the expression of MPO in both these populations it was shown that after stimulating the cells with LPS, the smaller neutrophils (FSC*low*) (84.08 %, $P = 0.0036$) displayed higher expression of MPO in comparison to the larger cells (FSC*high*) (21.71 %). Similar results were observed for the stimulation with fMLP, i.e., the fraction of MPO-positive FSC*low* neutrophils increased (69.95 %, $P = 0.0078$) in comparison to FSC*high* neutrophils (4.54 %) (Fig. 5). Separate populations in terms of size were not identified after stimulation with the test cytokines at the same incubation periods.

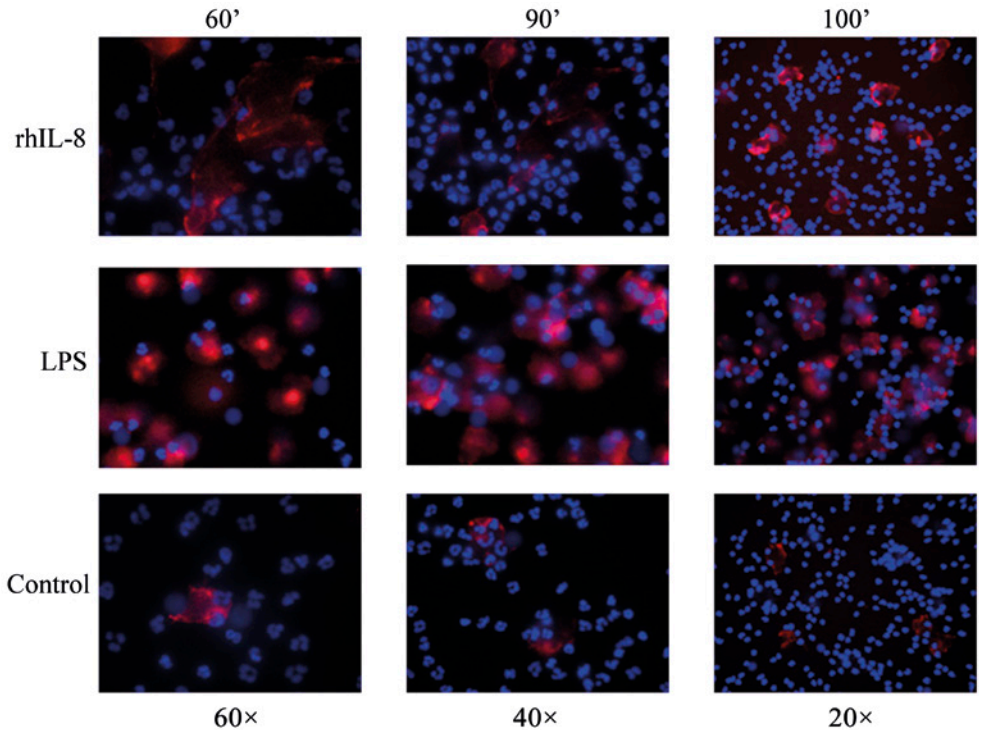

Fig. 1. Microscopic photographs of NETs after IL-8 and LPS stimulation in different periods of time (60'; 90'; 120') and different zoom (60×, 40×, 20×)
Blue colour – staining of total DNA (Hoechst 33342), red colour – staining of myeloperoxidase (Clone 8E6)

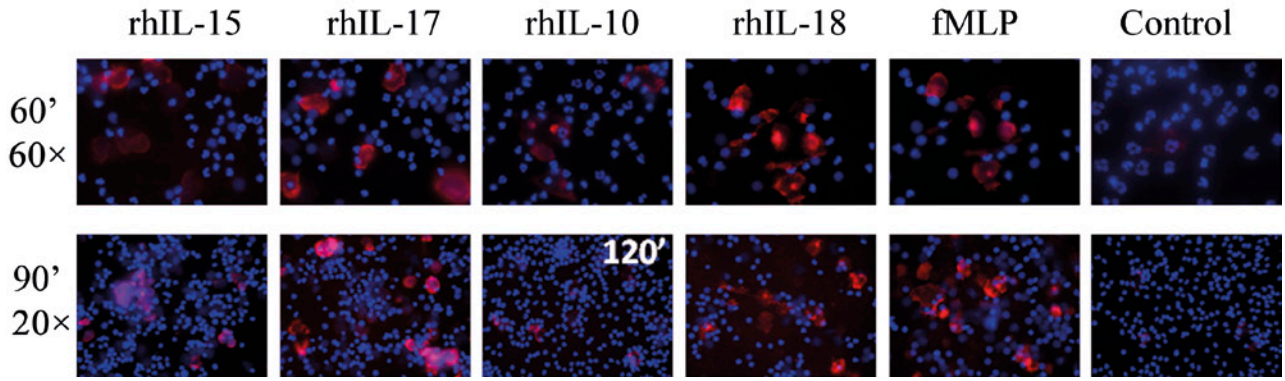

Fig. 2. Microscopic photographs of NETs after IL-10, IL-15, IL-17, IL-18, fMLP stimulation in different periods of time (60'; 90'; 120' – only for IL-10) and different zoom (60×, 20×)
Blue colour – staining of total DNA (Hoechst 33342), red colour – staining of myeloperoxidase (Clone 8E6)

Table 2. Percentage of cells forming NETs after stimulation

Percentage of neutrophils forming NETs [%]								
Time [60']	**PMNs**	**PMNs+IL-8**	**PMNs+IL-15**	**PMNs+IL-17**	**PMNs+IL-18**	**PMNs+IL-10 [120']**	**PMNs+LPS**	**PMNs+fMLP**
$\bar{X}$	1.27	13.4*[a]	12.93*[a]	15.13*[a]	14.2*[a]	8.73*[ab]	38.93*	16.6*[a]
SD	1.03	1.68	2.15	2.88	2.3	1.53	4.15	2.06

Statistically significant difference ($P < 0.001$)
* – between PMNs and PMNs incubated with stimulators
[a] – between PMNs incubated with LPS and the other groups of cells
[b] – between PMNs incubated with IL-10 and the other groups of cells

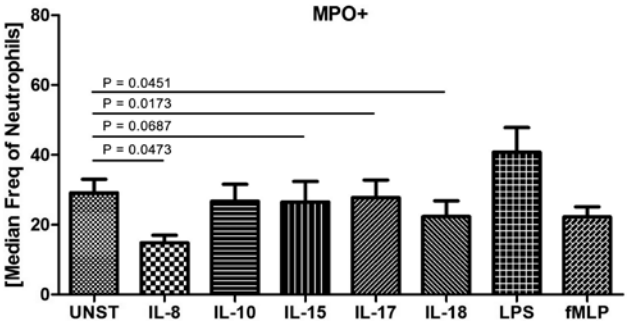

Fig. 3. Evaluation of neutrophil MPO content with flow cytometry, unstimulated and stimulated cells

Evaluation of cfDNA

Table 3 shows measurements of cfDNA in the supernatants of neutrophils incubated with various stimulators.

Discussion

The discovery of NETs provoked a myriad of questions connected with this phenomenon and made it necessary to reevaluate the functions of neutrophils in their natural environment, which contains, among other, cytokines (Brinkmann et al., 2004; Lögters et al., 2009; Papayannopoulos and Zychlinsky, 2009; Mantovani et al., 2011).

There are known reports indicating that cytokines, apart from their direct influence on the function and lifespan of neutrophils, may be involved in the formation of neutrophil traps. There are studies indicating active creation of NETs under the influence of pro-inflammatory cytokines: IL-8, IFN or GM-CSF with C5a (Brinkmann et al., 2004; Fuchs et al., 2007).

Our own research showed that other cytokines as well, i.e., IL-17, IL-15 and IL-18, are capable of modulating the functions of neutrophils, inducing formation of neutrophil extracellular traps to a similar extent as IL-8.

Earlier studies indicated a pro-inflammatory character of the studied cytokines (Fehniger and Galigiuri, 2001; Leung et al., 2001; Garley et al., 2010; Lin et al., 2011). The release of extracellular neutrophil traps in response to IL-17, IL-15 or IL-18 may also intensify the inflammatory reaction. The components of NETs are mainly formed of nuclear and cytoplasmic proteins that have strong antibacterial and/or immunomodulating properties (Brinkmann et al., 2004). Abnormal amounts of the neutrophil traps or lack of DNase, which degrades the traps, contribute to extending the duration of NETs.

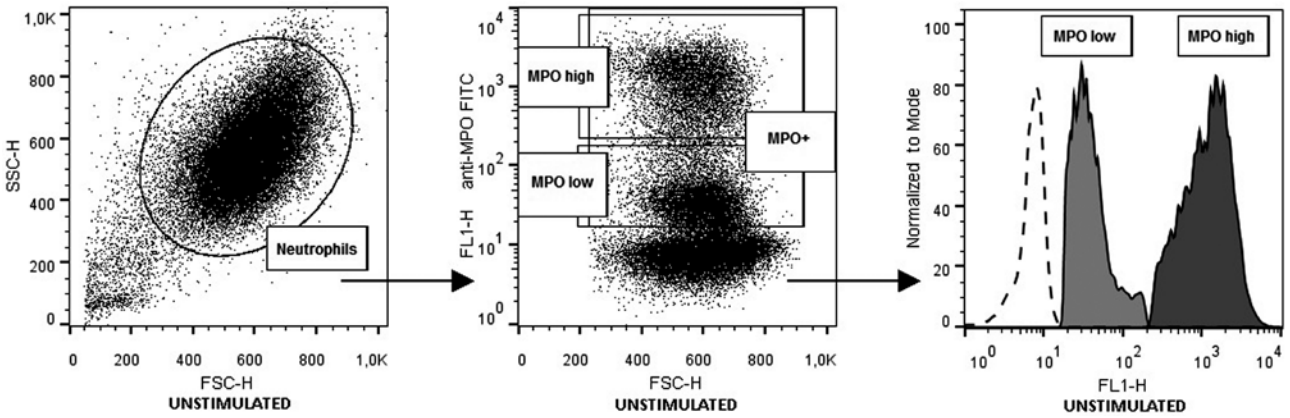

Fig. 4a. Two populations of neutrophils differing in MPO expression (MPO*low* and MPO*high*) – gating strategy

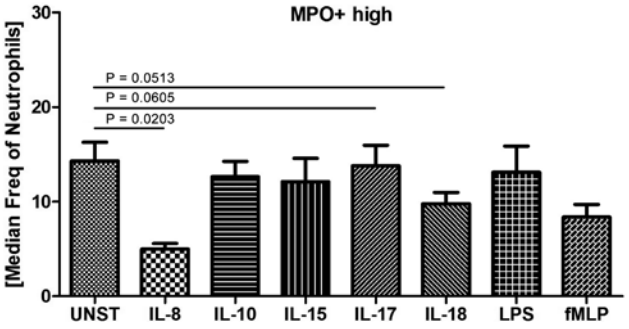

*Fig. 4b***.** Fraction of MPO*high* neutrophils, results of flow cytometry

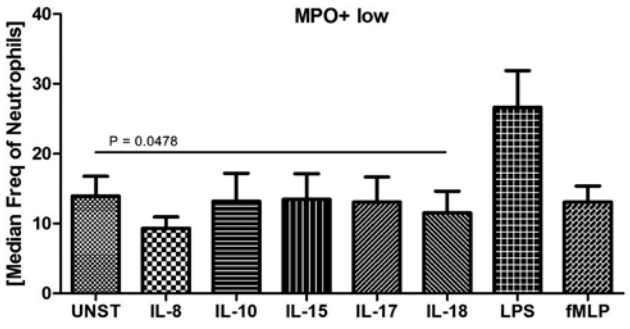

Fig. 4c. Fraction of MPO*low* neutrophils, results of flow cytometry

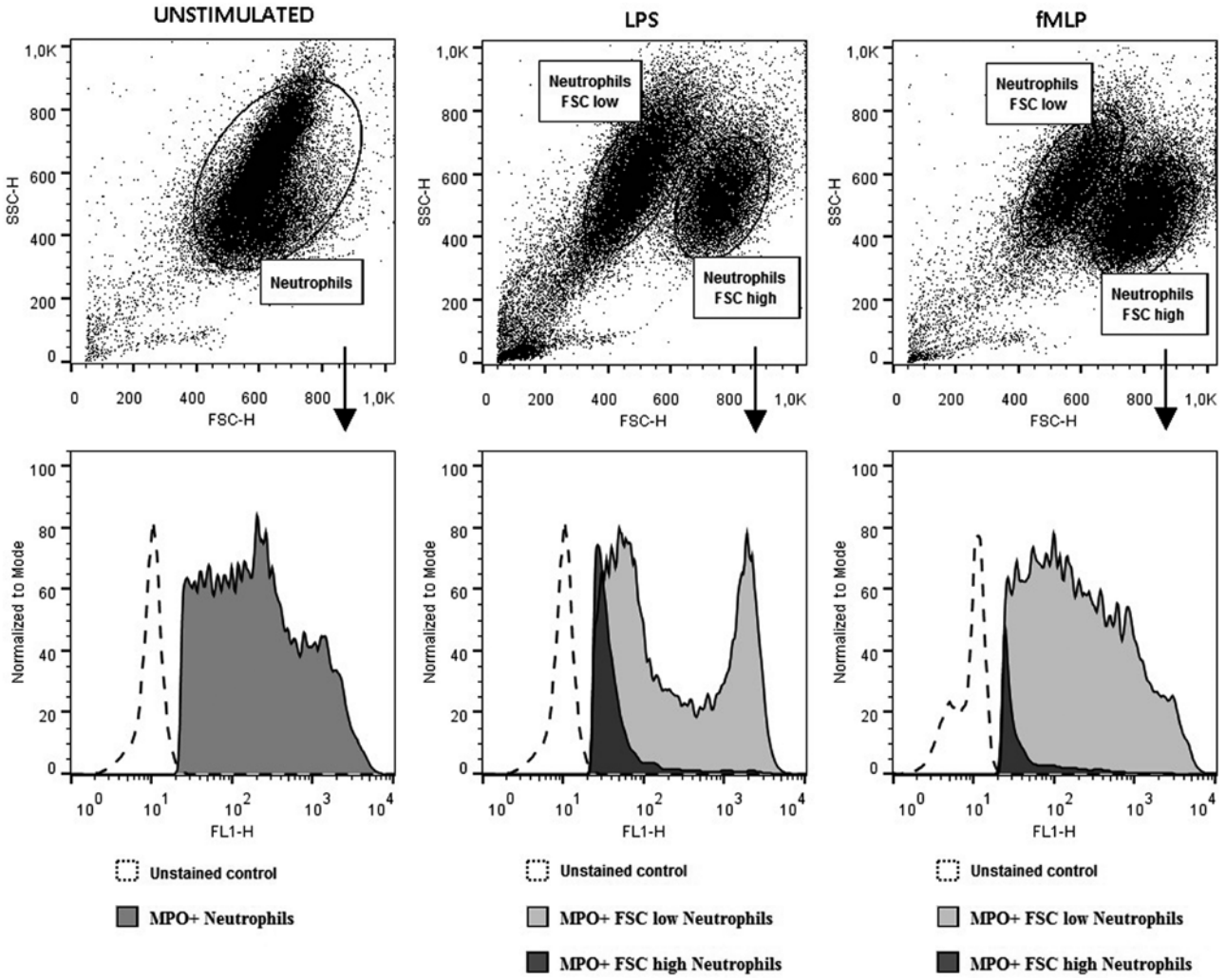

Fig. 5. Two populations of neutrophils significantly differing in size (FSC*high* and FSC*low*) observed in flow cytometry

Table 3. cfDNA concentrations in cell supernatants

cfDNA [ng/µl]								
Time [60']	**PMNs**	**PMNs+IL-8**	**PMNs+IL-15**	**PMNs+IL-17**	**PMNs+IL-18**	**PMNs+IL-10 [120']**	**PMNs+LPS**	**PMNs+fMLP**
$\bar{X}$	147	194*[a]	181[a]	197*[a]	175[a]	153[a]	263*	179[a]
SD	26.4	18.9	23.6	22.9	21.2	34.2	39.1	31.8

* – between PMNs and PMNs incubated with stimulators
[a] – between PMNs incubated with LPS and the other groups of cells

Long-lasting traps may physically clog microvessels, hamper blood circulation, or damage tissues enzymatically, recruit other cells, and strongly promote inflammation. On the other hand, there is also the risk of an autoimmune reaction. Chronic and lasting NETs induce production of ANA and ANCA antibodies (Hakkim et al., 2010; Patel et al., 2010).

Interesting is the fact that the formation of NETs was also stimulated with anti-inflammatory cytokine IL-10. The microscopic image of neutrophils stimulated with this cytokine showed delayed formation of the traps and their concentration around the cell, in comparison to PMNs stimulated with IL-8, whose traps exhibited more of a spatial character.

A clustered trap ensures high local concentration of antibacterial factors, allowing effective killing of pathogens, whereas when the NETs take up more space, it allows them to capture bacteria more effectively and gives better control of the infection (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2012).

The variety in the observed images of neutrophil traps produced in response to the studied cytokines may be associated with the manner in which the traps are released from the cells. It is known that cells may use catapult-like release of NETs in order to increase their spatial range (Lu et al., 2012). Such picture was observed after stimulating the cells with IL-8. On the other hand, the long strands observed during the incubation of neutrophils with IL-18 may result from gradual release of the trap from the cell by exocytosis and formation of strands. By contrast, the concentration of the neutrophil trap around the cell after stimulation with IL-10 may be the result of releasing the cell content upon its altruistic death. This spatial limitation of the trap is associated with minimal damage to the host cell, which may be confirmed by the anti-inflammatory function of IL-10.

The formation and release of NETs containing myeloperoxidase observed in the microscope is probably the result of the decreased content of neutrophil MPO indicated in the cytometric test (Parker and Winterbourn, 2013). The existence of two neutrophil populations with differing expression of MPO (MPO*low* and MPO*high*) allows assuming that the low expression of myeloperoxidase in the studied PMNs is connected with the decreased fraction of MPO*high* neutrophils that underwent NETosis.

Bacterial products seem to be the strongest stimulants of NET release (Brinkmann et al., 2004; Fuchs et al., 2007). This is confirmed by the microscopic image of neutrophils stimulated with LPS or fMLP, which demonstrated the largest amounts of traps when compared to NETs induced with interleukins.

The results of cytometric tests showed that after stimulation with LPS or fMLP, there were two neutrophil populations that varied in size (FSC*low* and FSC*high*), which was not observed in the case of cytokines. It was also found that the smaller neutrophils (FSC*low*) showed higher concentrations of MPO than the larger neutrophils (FSC*high*) after stimulation with exogenous factors, which may indicate that cells lose MPO during active release of the traps from live cells.

Identification of neutrophils that differ in MPO expression (MPO*low* and MPO*high*) and size (FSC*low* and FSC*high*) seems to confirm the existence of yet other subpopulations within the population of cells that has hitherto been considered homogeneous.

Research done by other authors has demonstrated the existence of two subpopulations of mouse neutrophils with different action against *Staphylococcus aureus*. Subpopulations of human neutrophils with a specific phenotype were found demonstrating expression of HLA-DR, CD80, and CD49d and having a greatly prolonged, over 72 h, lifespan. Denny et al. (2010) discovered a characteristic, pro-inflammatory subpopulation of neutrophils called low-density granulocytes (LDGs) in patients with systemic lupus erythematosus (Chakravarti et al., 2009).

The results of the determination of free circulating DNA in the supernatants of stimulated neutrophils confirmed the microscopic observation and the cytometry results. These observations indicate the possibility of using cfDNA as a marker of the NET release.

Conclusions

The existence of two separate subpopulations of neutrophils, increased cfDNA and the different amounts of NETs observed in the stimulated cells is likely to be related to the presence of cytokines in their micro-environment. Precise identification of the factors regulating the formation of NETs may contribute to learning the pathomechanism of diseases that involve neutrophils in their pathogenesis. The obtained results may prove useful in diagnosing and monitoring these disorders. Moreover, further research will allow exploration of the population of human neutrophils.

Disclosure of conclict of interest

The authors confirm that they do not have any conflicts of interest.

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