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Influence of Extracellular Traps (ETs) on the Differentiation of TCD4 Cell Profiles and Macrophages in Human Autologous Culture

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ABSTRACT

Background: **The formation of extracellular traps (ETs) as a microbicidal functional mechanism of various leukocytes has taken on importance today and they are implicated in the pathogenesis of various diseases and have even been involved in the current pandemic.**

Objectives: **Study ETs generated *in vitro* from healthy human blood leukocytes against different stimuli (LPS, fMLP) and their influence on different T cells profiles and monocyte-macrophages, in autologous cell cultures.**

Methods: **Heparinized human blood samples were collected with ethical consent. ETs generation was performed by stimulation with LPS and fMLP. Subsequently they were isolated. ETs influence on cell profile differentiation was performed in samples without stimulation, with OVA addition samples, and OVA-ETs addition samples. This assay was observed through immunofluorescence (IF) labeling of molecules of T CD4 profile; Th17 and innate lymphoid cells 3 ILC3 by RORɣ; activation status of T cells by CD45RO; and M1 macrophage profile by iNOS.**

Results: **Significant CD4 and CD45RO positive cells percentage were observed between paired control samples and OVA addition samples ($p < 0.05$), between paired control samples and OVA-ETs addition samples ($p < 0.05$). In an independent experiment, significant differences were observed between OVA addition samples vs. OVA-ETs addition samples ($p < 0.05$). At 72 h of culture, no significant differences were found between the paired samples in any case. There were no significant differences between paired control samples and OVA addition samples or OVA-ETs samples, neither 24 and 72 h of culture in RORɣ positive cells percentage or iNOS positive cells percentage.**

Conclusions: **Influence of ETs on T cell activation was observed and components of autologous ETs did not elicit classical activation of M1 macrophages**.

Keywords: Extracellular traps, ETs, NETs, human autologous leucocytes.

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I. Introduction

Extracellular traps (ETs) are structures composed of chromatin, histones and granule proteins and were first described in polymorphonuclear (PMN) neutrophil leukocytes by Brinkmann et al [1] calling them neutrophil extracellular traps (NETs). It is currently known that cells other than neutrophil PMNs are capable of generating ETs [2], for example, mast cells, monocytes, tissue macrophages and eosinophils [2]-[4]. ETs formation has even been described in an in vitro study of lymphocytes challenged with phorbol myristate acetate (PMA) and serum from systemic lupus erythematosus (SLE) patients [5]. ETs constitute a new defense mechanism of the immune system in response to various microorganisms and other stimuli [1].

DNA present in eosinophilic extracellular traps (EETs) was reported to be of mitochondrial and nuclear origin [6]. In EETs, intact granules release to the extracellular space have been described, which are functionally competent. Therefore, EETosis results in extracellular nuclear DNA networks generation with histones and cell-free granules, both of which can exert biological activities for postmortem eosinophils [7].

NETs are made up of DNA, histones and granular proteins that are released outside the PMN, which bacteria [8], fungi [9], viruses [10] and parasites entrapment [11]. NETs fibrous structure is necessary for sequestration and destruction of bacteria, by delivering a high local concentration of molecules, such as degenerative extracellular proteases that degrade bacterial virulence factors by inhibiting neutrophil phagocytosis, an example of these is neutrophil elastase (NE).

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Furthermore NETs granular proteins can prevent potentially harmful proteins such as proteases from diffusing and inducing damage adjacent tissue to inflammation site [1].

NETs components are still under study and approximately 30 constituent proteins have been described [12], [13]. In the laboratory where this work was carried out, finding of costimulatory molecules B7 CD80 and CD86 in the NETs was described [14], [15], as well as beta tubulin and Major Histocompatibility Complex Class II (CMH II) HLA-DR presence [16].

Regarding the formation of NETs, in stimulated cells, development of these traps begins with chromatin decondensation simultaneously with loss of nuclear structure. Nuclear membrane and granule membrane disintegrate resulting in nuclear, cytoplasmic and granular contents mixture, which is finally extruded into the extracellular space generating ETs [17]. In this process, elastase migrates from azurophilic granules to the nucleus and partially degrades histones, promoting chromatin decondensation. MPO synergizes this action. After 1 hour, nucleus and cytoplasm components are mixed. Finally, plasma membrane ruptures and expels interior of the cell into extracellular space, forming NETs. Two mechanisms for NET release in response to various stimuli are described: 1. rapid and early release (5 to 60 minutes after in vitro stimulation with Staphylococcus aureus or lipopolysaccharide (LPS) and 2. slow (2 to 4 hours) leading to the death of the neutrophil, the PMN "NETosis" [18].

NETosis can be induced by physiologically relevant stimuli and the participation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex and the formation of reactive oxygen species (ROS) is also very diverse in the signaling pathways used. Furthermore, outside of the NADPH-oxidase complex, the neutrophil has other sources of ROS that are sufficient to induce NETosis. This indicates that in different situations NETosis depends on this enzyme complex and in others it does not [19].

It has been observed that NETs are potential sources of autoantigens, which contribute to the development of autoimmune pathologies, such as systemic lupus erythematosus, rheumatoid arthritis, among others [20]. They have also been linked to the development of thrombotic diseases and cancer [21]. Impaired NET formation can be a critical facet of an immunodeficiency. The NET escape mechanism may be one of the virulent factors in invasive infections [8].

Patients with SLE, rheumatoid arthritis (RA), and vasculitis associated with antineutrophil cytoplasmic antibodies (AAV) develop autoantibodies that recognize the components of NETs: double-stranded DNA, citrullinated proteins, and components of azurophilic granules, respectively. These antibodies can arise from prolonged exposure to NETs due to excessive production or reduced ability to degrade NETs. In fact, hereditary deficiencies in DNases lead to pediatric SLE. Furthermore, uptake of NETs by antigen-presenting cells drives autoimmunity in an RA model where NET-loaded fibroblasts stimulate the production of anti-citrullinated peptide antibodies. Anti-NET autoantibodies also inhibit NET degradation, aggravating the disease. As the disease progresses, immune complexes with NET components are found in glomerulonephritis, a common sequela of SLE and AAV [21].

With regard to the current health situation of the pandemic recorded by the 2019 coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), it is important to note that we also see NETs influence in their pathogenesis development. In this case, the virus-induced NETosis process could operate as a double-edged sword: on the one hand, there are essential and efficient mechanisms to trap the virus, and on the other, there are high-intensity immunological and inflammatory processes triggered by the release of NETs that cause damage to the body. These interactions could influence the symptoms of COVID-19, the relationship between hyperinflammation (overproduction of NETs and cytokine storm) and PMNs neutrophil function to destroy the viral infection [22]. Excessive PMN neutrophils in peripheral blood counts and excessive NETs have been reported in severe COVID-19 development, as well as high pulmonary neutrophilic infiltration as a pathological finding in autopsies of patients who died of COVID-19-associated pneumonia [23]. In a recently published review paper, role of NET formation in COVID-19 pathogenesis and the consequent occurrence of thromboembolic phenomena and their even greater severity in patients with previous comorbidities such as cardiovascular and metabolic diseases are emphasized [24]. The importance of NETs impact in the course of severe COVID-19 is emphasized and research towards new therapeutic strategies that regulate extracellular traps formation is promoted [24].

Interactions of ETs and antigen presenting cells (APCs): functional consequences of ETs in the interaction with immune cells have not been studied in depth. Few studies have evaluated the potential effects of NETs on APCs, some were pro-inflammatory effects, and others anti-inflammatory [25]. In an in vitro study, mitochondrial damage has been observed in macrophages and dendritic cells exposed to NETs [26]. There are works that report NETs as negative regulators of the activation induced by LPS in dendritic cells derived from monocytes [25] and other authors instead propose them as activators of macrophages [27].

In relation to neutrophils and macrophages interactions, PMNs contribute to the activation and recruitment of macrophages at the site of infection or in acute inflammation [28]. There is evidence for the existence of subsets of neutrophils that show a unique pattern of cytokine and chemokine production and differ in the expression of Toll-like receptors (TLRs) and integrins. It is possible that these “pro-inflammatory” and “anti-inflammatory” forms of neutrophils change the course of the immune response by inducing classically activated M1 and alternatively activated M2 macrophages, respectively [29]. Different neutrophil phenotypes have been described [30], although the parameters, methods, species, tissues, and biomarker molecules are different. Anyway, these studies are very useful in the developed context avoiding extrapolation.

Regarding interactions between NETs and lymphocytes a role for NETs in T cell sensitization has been proposed [31]. It has been observed that NETs released by neutrophils produce sensitization of T cells, reducing their activation threshold. NET-mediated sensitization increases the T cell

response to a specific antigen even if the stimulus is suboptimal and requires contact between the T cell and NETs and signaling through the TCR [31].

The release of B7 molecules by NETs that was recently described could influence the cellular environment, by activating the costimulation pathway B7-1 B7-2 CD28 CTLA-4, which results in stimulation or inhibition of immune response depending on various subpopulations of surrounding naïve, effector, or memory T cells [14], [15]. Activated effector and memory T cells express the marker CD45RO on their surface [32].

For all of the above, to provide new knowledge on the subject, the study of ETs influence on TCD4 cell profiles differentiation and human macrophages in autologous culture is proposed. ETs could influence cellular environment due to the content they release, producing activation of immune response and differentiation of CD4 T lymphocytes and macrophages from monocytes. Th17 cell profile and M1 macrophages would be stimulated.

II. Material And Methods

A. Samples

Heparinized human blood samples (n = 10) were collected with ethical consent according to procedures approved by ethical committee of National Hospital Clinicas. Samples donated by the Blood Bank, Institute of Hematology and Hemotherapy of the National University of Cordoba in anonymity, with negative serology: Hudleson (Wiener), VDRL (Wiener), Chagas HAI (Wiener) Chagas EIE (Biomerieux), HBs EIE (Biomerieux), HBc (Biomerieux), HCV EIE (Murex), HIV Ac EIE (Biomerieux), HIV Ag EIE (Biomerieux), HTLV EIE (Murex).

B. Autologous Cultures

Blood samples obtained by the method already described above were used to culture leukocytes at 37 °C in TC199 medium (Sigma, St. Louis, MO) supplemented with L-glutamine (Sigma, St. Louis, MO), added with filtered serum from the same donor. A classic cell viability test was performed by Trypan Blue exclusion at 0.5%. All cell cultures were prepared under sterile conditions under a hood equipped with ultraviolet light and laminar flow. A 24-well cell culture plate was prepared by putting a sterile 13 mm round glass cover slip into each well.

C. ETs Generation

For ETs generation, were used: LPS, Lipopolysaccharides of *Escherichia coli* (Sigma-Aldrich) 25 ng/ml and fMLP, formylated peptides, N-formyl-met-leu-phe (Sigma-Aldrich) 0.25 ng/ml. Cultures were sampled at 30 minutes to observe the occurrence of ETs. The released ETs were visualized by fluorescence microscopy using DAPI (4,6'-diamino-2-phenylindole) (Sigma, St Louis, MO) for DNA staining.

D. ETs Isolation

After stimulation with fMLP or LPS for ETs generation, culture plate was gently aspirated and the aspirate was discarded, leaving ETs layer and leukocytes adhered to the bottom. The bottom of the plates was washed using cold PBS without Ca and Mg by pipetting. Solution obtained from the washing was collected in a 15 ml conical tube and centrifuged for 10 minutes at 450 g at 4 °C. Leukocytes settled to the bottom, leaving a cell-free ET-rich supernatant. Supernatant was divided into 1.5 ml tubes and microcentrifuged for 10 minutes at 18,000 g at 4 °C. The supernatant was discarded, and the pellet obtained was resuspended in cold PBS at 4 °C. This produced the cell-free ETs stock [33].

E. Challenge Assay with Heterologous Ovalbumin Antigen (OVA)

A final concentration of 100 µg/ml of OVA was used from culture time zero. Samples were taken at 24 and 72 hours from paired autologous culture, with and without OVA (controls).

F. ETs Influence Assay on Cell Profile Differentiation

Paired autologous total leukocytes cultures with and without (controls) OVA heterologous antigen challenge were subjected with the cell-free ET stock according to the ET isolation assay. Samples of these cultures were taken at 24 and 72 hours to process with IF and mark the different profiles.

G. Immunofluorescence (IF)

Cells culture were washed briefly in PBS (phosphate buffered saline), fixation was performed with 4% paraformaldehyde for 10 minutes and washed in three changes in PBS. It was incubated with 5% blocking serum albumin in PBS to prevent non-specific staining for 20 minutes. It was washed with PBS. It was incubated with antibodies (Ab) anti-CD45RO (FITC; Santa Cruz Biotechnology) and anti-CD4 (PE; Santa Cruz Biotechnology) at 4º ɵ C overnight. It was washed with PBS and nuclear staining with DAPI (4,6'-diamidino-2-phenylindole) (Sigma, St Louis, MO). Other samples were incubated with primary antibody anti-NOS2 (C-11) (Santa Cruz Biotechnology) or with anti-RORɣ (D-4) (Santa Cruz Biotechnology) for 1 hour at 37 °C, then washed with PBS and dried. incubated with secondary anti-mouse antibody (m-IgG BP-PE, phycoerythrin conjugated; Santa Cruz Biotechnology) at 4 °C overnight. It was washed with PBS and nuclear staining with DAPI (4,6'-diamidino-2-phenylindole) (Sigma, St Louis, MO). Samples were mounted with 90% glycerol in PBS. The observation of preparations was carried out in Axioscop 20, MC80, trinocular, Carl Zeiss videomicroscope.

H. Positive IF-labeled Cells Quantification

Quantification of positive IF-labeled cells in samples of human total leukocytes in autologous cultures in paired samples: the percentage of positive cells was calculated as the mean value in three fields (1000x) normalized by the total number of cells visualized with DAPI nuclear staining. Data were expressed as mean value ± SD. FIJI software was used [34].

I. Statistical Analysis

Data were analyzed with t-test for paired samples. The statistical program Infostat was used for its analysis [35] $p<0.05$ was considered statistically significant.

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III. Results

A. ETs Generation and Isolation. ETs generated by stimulation with LPS or with fMLP, in autologous cultures of total human blood leukocytes

Autologous cultures of total human blood leukocytes were prepared and stimulated to generate ETs with: 1. fMLP or 2. LPS. ETs generated were visualized with fluorescence microscopy using DAPI for DNA staining. ETs were observed with DAPI as a diffuse staining or fibrillar appearance. ETs isolation was carried out following the technique of [33]. After stimulation with fMLP or LPS for the generation of ETs, this produced the cell-free stock of ETs. (Fig. 1)

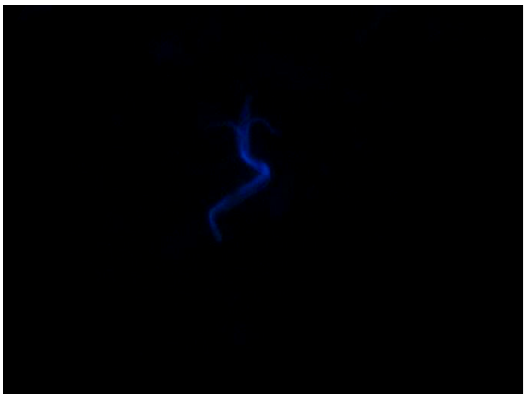

Fig. 1. ET generated by stimulation with LPS. Representative image of IF, cell-free stock from paired control samples of autologous total leukocyte culture stimulated with 25 ng/ml LPS. 1000x. DAPI staining.

B. ETs Influence on Cell Profiles Differentiation in Autologous Cultures Stimulated with Heterologous OVA Antigen

Expression of CD4 and CD45RO in leukocytes stimulated in vitro with ETs, OVA or OVA-ETs: paired autologous cultures of total leukocytes unchallenged (controls) and challenged with heterologous antigen OVA were submitted with the stock of isolated ETs.

The IF technique for CD4 and CD45RO was performed on paired culture samples of total leukocytes at 24 h of culture, without stimulation (controls) and stimulated with OVA and ETs generated with fMLP. This was done in two independent experiments (Fig. 2). In one of the paired 24 h autologous culture leukocyte samples (Fig. 2), numerous leukocyte nuclei are visualized, stained with DAPI, positivity for CD45RO (green) and CD4 (red) is observed in a higher percentage of cells (76.24%) in the samples stimulated with OVA and subjected to the stock of ETs (Fig. 2m). Significant differences are observed between paired control vs. OVA addition samples ($p<0.05$) and between paired control vs. OVA-ETs addition samples ($p<0.05$). There were no significant differences between paired control samples vs. ETs addition only and neither when comparing OVA addition samples vs. OVA-ETs samples (Fig. 2m).

In other independent experiment, with blood from another donor, in paired culture samples of leukocytes without stimulation (controls) and stimulated with OVA and ETs at 24 h of culture significant differences ($p<0.05$) were observed between control samples vs. OVA-ETs addition and between those stimulated with OVA vs. OVA-ETs addition (Fig. 2n).

IF technique for CD4 and CD45RO was performed on paired samples of autologous cultures of total leukocytes at 72 hours of culture. Expression of CD4 and CD45RO was observed in paired culture samples of total leukocytes without stimulation (controls) and those stimulated with OVA and LPS-generated ETs (Fig. 3). When comparing the percentages of positive cells for CD4 and CD45RO of the paired control samples with the samples with added ETs or stimulated with OVA or with OVA-ETs, there were no significant differences between the samples at 72 h of culture. (Fig. 3p). On the other hand, in the experimental situation where no stock of ETs was added but stimulation with OVA was performed, the generation of ETs was observed (Fig. 3j).

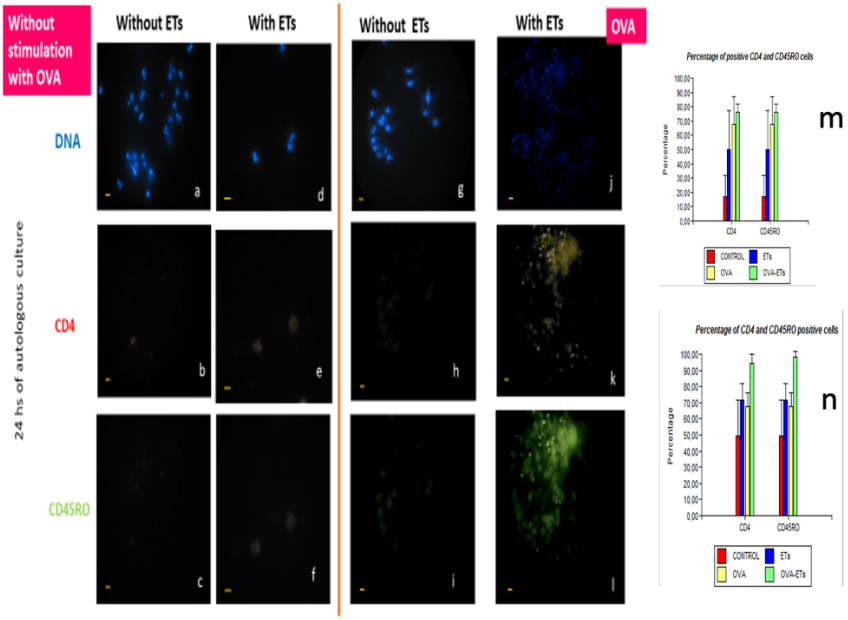

Fig. 2. Expression of CD4 and CD45RO in paired culture samples of autologous total human leukocytes without stimulation (controls) and stimulated with OVA (ovalbumin) and ETs (extracellular traps). 24 h of culture. Representative immunofluorescence microscopy images. ETs generated with fMLP. a-f: without stimulation with OVA (ovalbumin). g-l: with stimulation with OVA. a-c, g-i: no aggregate of ETs. d-f, j-l: with added ETs. DNA (blue) and expression of CD4 (red) and CD45RO (green) are observed. 1000x. Scale bar represents 10 µm. m, n: percentage of CD4 and CD45RO positive cells in paired samples of autologous human total leukocyte cultures. 24 hours of culture. These graphics represent two independent experiments. Data are presented as mean value ± SD. Student's t-test ($p<0.05$) was performed for paired samples.

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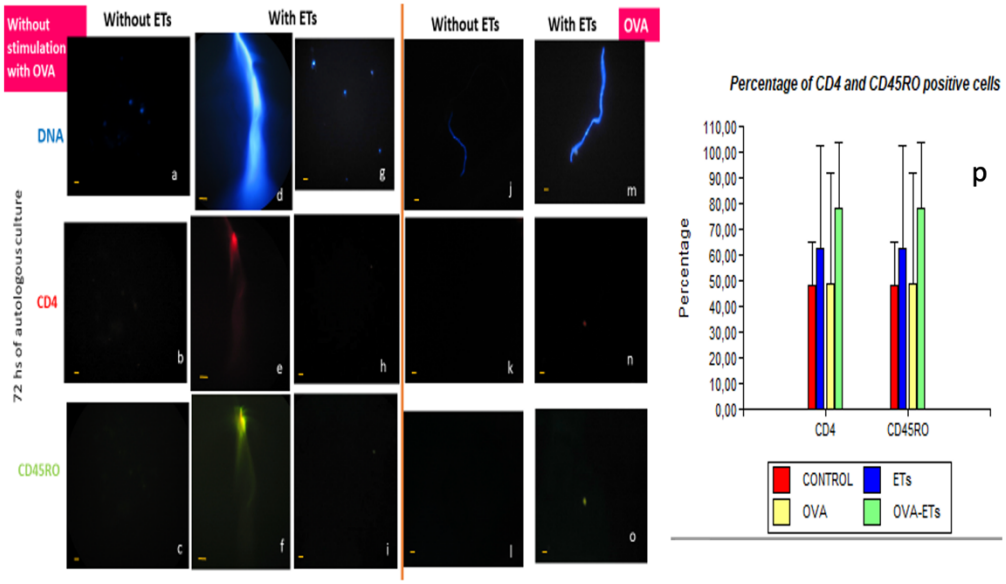

Fig. 3. CD4 and CD45RO expression in paired autologous total leukocyte culture samples without stimulation (controls) and stimulated with OVA (ovalbumin) and ETs (extracellular traps). 72 hours of culture. Representative images of immunofluorescence microscopy. ETs generated with LPS. a-i: without stimulation with OVA (ovalbumin). j-o: with OVA stimulation. a-c, j-l: without addition of ETs. d-i, m-o: with addition of ETs. DNA (blue) and expression of CD4 (red) and CD45RO (green) are observed. 1000x id: 400x. The scale bar represents 10 µm. p: percentage of CD4 and CD45RO positive cells in paired samples of autologous human total leukocyte cultures. 72 hours of culture. The data are presented in mean value ± SD. Student's t test ($p<0.05$) was performed for samples paired.

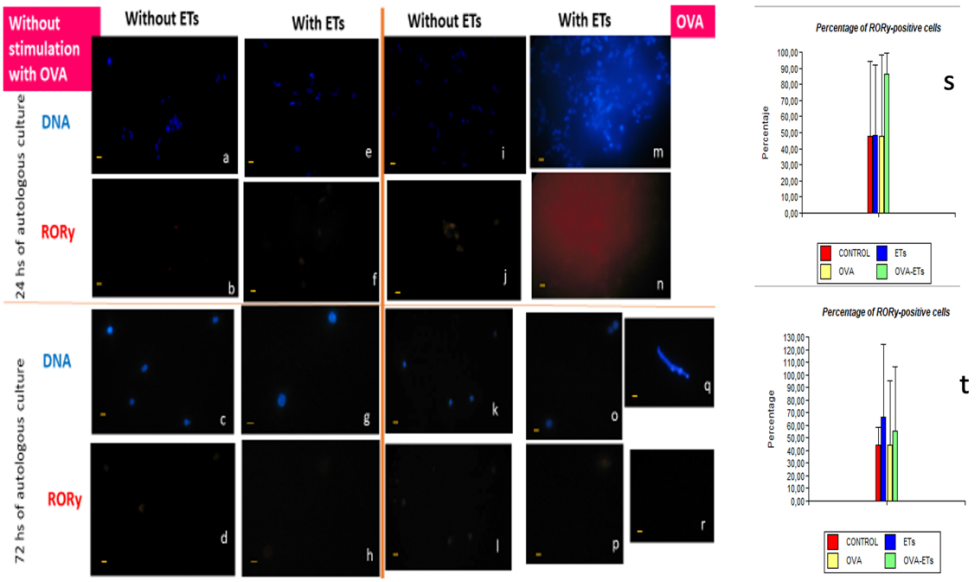

Fig 4. RORγ expression in paired leukocyte culture samples unstimulated (controls) and OVA-stimulated total autologous humans (ovalbumin) and ET (extracellular traps). 24 and 72 hours of culture in independent experiments. Representative microscopic images of IF. a–h: without OVA stimulation. i-r: with OVA stimulation. a, b, e, f, i, j, m, l: 24 hours of culture. c, d, g, h, k, l, o-r: 72 hours of culture. e,f: ET generated with fMLP. g, h, m-r: ETs generated with LPS. DNA is observed (blue) and expression of RORγ (red). 1000x. The scale bar represents 10 µm. s: percentage of RORγ-positive cells in paired culture samples of autologous human total leukocytes. 24 hours of culture. t: percentage of RORγ-positive cells in paired culture samples of autologous human total leukocytes. 72 h of culture. The data are presented in value mean ± SD. Student's t test ($p<0.05$) was performed for paired samples.

Expression of RORγ in leukocytes stimulated in vitro with ETs, OVA, or OVA and ETs. Paired autologous cultures of total leukocytes without challenge (controls) and stimulated with heterologous antigen OVA were submitted with the stock of isolated ETs. IF technique for RORγ was performed on paired samples of autologous cultures of total leukocytes at 24 and 72 hours of culture, in independent experiments. (Fig. 4). Numerous leukocyte nuclei are visualized in the samples in Fig. 4, stained with DAPI and positive for RORγ (red). When comparing the percentages of positive cells for RORγ of the paired control samples with the samples with ETs addition or stimulated with OVA or OVA-ETs, there were no significant differences between the samples, both at 24 and 72 h of culture. (Fig. 4s, t).

Expression of iNOS, M1 macrophage profile molecule leukocytes stimulated in vitro with ETs, OVA or OVA-ETs: paired autologous cultures of total leukocytes with and without challenge (controls) with heterologous OVA antigen were submitted with the stock of isolated ETs. The IF technique for iNOS was performed on paired control samples of autologous cultures of total leukocytes, with ETS addition, OVA addition, and OVA-ETs addition at 24 and 72 hours of culture. (Fig. 5). When comparing the percentages of positive cells for iNOS of the paired control samples with the samples with added ETs or stimulated with OVA or with OVA and ETs, there were no significant differences between the samples, both at 24 and 72 h of culture (Fig. 5s).

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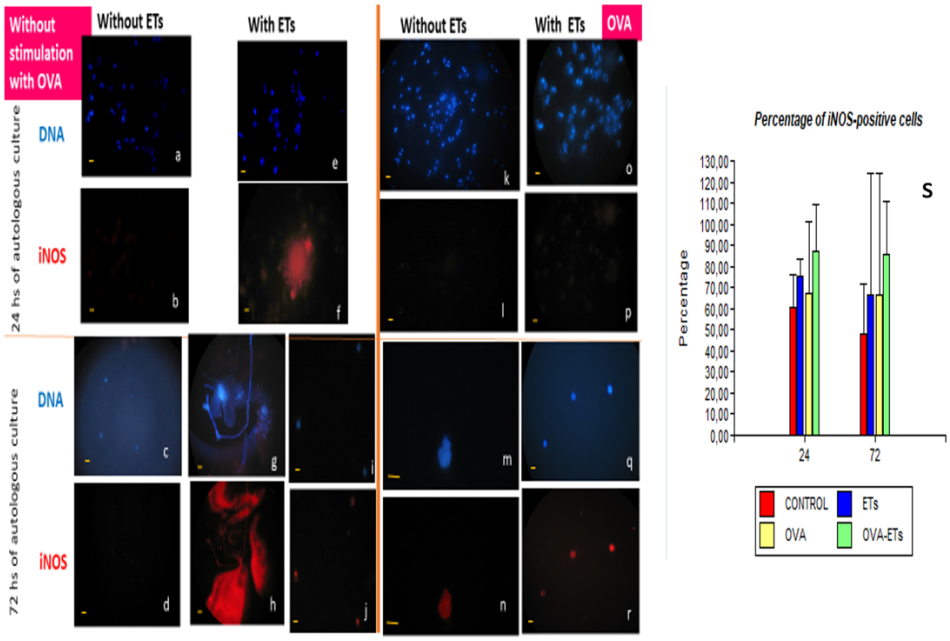

Fig.5. iNOS expression in paired cultured samples of autologous human total leukocytes without stimulation (controls) and stimulated with OVA (ovalbumin) and ETs (extracellular traps). 24 and 72 hours of culture in independent experiments. Representative images of immunofluorescence microscopy. 24 and 72 hours of culture. a-j: without OVA stimulation. k-r: stimulated with OVA. a, b, e, f, k, l, o, p: 24 hours of culture. c, d, g, h, i, j, m, n, q, r: 72 hours of culture. e, f, o-r: ETs generated with LPS. g-j: ETs generated with fMLP. DNA (blue) and RORγ expression (red) are observed. 1000x. k, l: 400x. The scale bar represents 10 µm. s: percentage of iNOS-positive cells in paired culture samples of autologous human total leukocytes. 24 and 72 hours of culture. The data are presented in value mean ± SD. Student's t test ($p<0.05$) was performed for paired samples.

IV. Discussion

The formation of ETs as a microbicidal functional mechanism of various leukocytes has become important today, especially in neutrophilic PMNs with their early described NETs. Such traps have been implicated in various pathologies including chronic inflammation, autoimmunity, and cancer [36] and in the current COVID-19 pandemic [22]-[24]. Despite a growing understanding of the mechanisms underlying NET formation, much remains to be elucidated. Previously we described the finding of co-stimulatory B7 CD80 and CD86 molecules colocalized in NETs [14], [15], as well as the presence of beta tubulin and Major Histocompatibility Complex molecules Class II (CMH II) HLA-DR [16].

In this work, a study was carried out on the influence of ETs generated in vitro from leukocytes against different stimuli and their interactions with different T cells profiles and monocyte-macrophages, in autologous human blood cell cultures. The influence of ETs on the differentiation of cell profiles was observed through the IF labeling of CD4 T profile molecules; Th17 and innate lymphoid cells 3 ILC3 (RORγ +); and CD45RO+ activation status.

As previously described, NETs are made up of DNA, histones, and granular proteins [1]. It is known that free DNA in the extracellular space is an important DAMP (damage-associated molecular pattern) that can bind to RRP just as PAMPs do. Due to these interactions, the activation of immune cells is triggered and the consequent production of cytokines and chemokines [37]. It has been reported that TLR9 receptor which captures free DNA can be expressed in lymphocytes and its commitment can enhance the proliferation and/or production of cytokines of T cells stimulated by the TCR [38], it could partly explain the increased percentage of activated T cells marked with CD45RO+ found in the experimental situations of this work (Fig. 2m, 2n). On the other hand, in another study, the lowering of the activation threshold of T cells in the presence of NETs and dendritic cells was observed, and this NET-mediated sensitization requires contact between T cells and these traps [31]. However, in this same study, NETs were generated with fMLP, where no T cell activation was obtained [31]. It is worth noting here the different experimental conditions, Tillack et al. used netotic neutrophil PMNs, as opposed to isolated NETs as used in this work.

In addition to DNA and histones, the enzymes NE and MPO are some of the granule proteins released in NETs [12]. It has been described that with the inhibition of NE and the blockade of MPO, CD4 T effector cells differentiation decreases, although authors suggest that the mechanism by which this happens has not yet been elucidated [39]. On the other hand, Griffith et al. describe in vitro T-cell proliferation due to MPO acting as an autoantigen, both in peripheral blood mononuclear cell samples from control patients and patients with systemic vasculitis [40]. Other authors also suggest in vitro expansion of NE-specific CD4 and CD8 T lymphocytes [41]. Also referring to NE, in vitro it was observed that dendritic cells treated with said enzyme cause them to differentiate into cells that secrete TGF-β, with the consequent increase in regulatory CD4(+) FOXP3(+) T cells [42]. So, ETs components that affect immune cells could explain the contribution to T cell activation, such as the results found in this scientific investigation.

CD45RO molecule is an activation state marker in effector and memory T cells [32], although, as mentioned above, in PMN neutrophils they have been expressed as unconventional CD4 and CD45RO molecules in some phenotypes [43]. The state of cellular activation in lymphocytes was indicated by positivity for CD45RO. In 24-h autologous cultures, a higher percentage of positive cells

for CD4 and CD45RO was observed with respect to the control in the samples stimulated with OVA and subjected to the ET stock (Fig. 2m) ($p<0.05$). Significant differences were also observed between samples stimulated with OVA and those stimulated with OVA and addition of ETs in an independent experiment (Fig. 2n). This could be inferred to occur due heterologous OVA antigen effect, its antigenic processing and presentation, and due to aggregated ETs effect that contribute to costimulatory B7 molecules presence in its composition [14]. As is known, the two signals required for the activation of naïve T cells consist of antigen recognition and costimulation [32]. Here at this culture time it is noteworthy that CD4 and CD45RO positive neutrophil PMNs can be found present. In 72-hour cultures, fewer cells are observed in the samples (Fig. 3), this could be explained by the natural apoptosis suffered by PMN neutrophils after 15 hours of culture [44], so that the most of leukocytes present are lymphocytes. When comparing the percentages of positive cells for CD4 and CD45RO of paired control samples with the samples with ETs addition or OVA addition or OVA-ETs addition, there were no significant differences at 72 h (Fig. 3p). However, the percentage of positive cells for CD4 and CD45RO was higher in the control sample and in those stimulated with the different treatments at 72 hours compared to what was observed at 24 hours, in independent experiments (Fig. 2m and 3p). This may be due time passage in which culture microenvironment varies affects cell phenotypes and their differentiation, as it is known [45].

It is known that depending on the stimulus with which the NETs are generated, their composition will be influenced [46], and therefore will cause different responses, for example, another work describes that NETs generated by LPS can induce Th17 cells from memory CD4 cells stimulated with CD3/CD28 and with monocytes presence. However, this does not happen if NETs are generated with PMA [47]. In results of this work, in case of NETs addition generated with LPS, RORγ marker molecule expression of Th17 profile in total leukocytes stimulated in vitro with ETs, OVA, OVA and ETs in paired autologous cultures did not show significant differences. (Fig. 4s,t) both at 24 and 72 hours of culture, in independent experiments. Th17 cells and innate lymphoid cells 3 ILC3 are RORɣ+. This transcription factor is the marker molecule of these lymphocytes characterized by proinflammatory cytokines IL17 production and is also a marker of the small proportion of innate lymphoid cells 3 (ILC3) [48]. Apoptotic neutrophil PMNs that emerge over time in culture are phagocytosed by macrophages and under these conditions secrete less IL 23 and this negatively impacts the differentiation of CD4 T cells towards the Th17 RORɣ + cell profile [49]. This could explain what was observed.

The localization of iNOS has been studied in macrophages and neutrophils, where it can be found as an active enzyme in small vesicles, in primary and tertiary granules, close to phagosomes attached to the actin cytoskeleton, or in mitochondria [50]. Therefore, the observed cells positive for iNOS could correspond not only to macrophages, but also to PMN neutrophils, in the 24-hour samples due to the shorter half-life that these granulocytes suffer from natural apoptosis and has been described in culture [44]. In a review work it is highlighted iNOS expression is not common in monocytes, however, it is also described that by manipulating these cells, contact with plastic containers, culture media and serum, their activation can be triggered and consequent expression of NOS2 [51]. On the other hand, it is noted that the results of different reported works are not comparable due to the different species where the studies are carried out and the diverse source of obtaining the cells, whether they are purified monocytes, or total mononuclear cells, and the presence of platelets or platelet-free assays [51].

It is also reported that iNOS expression is favored in the presence of IFN-gamma stimulation [52] and interestingly, a study in mice showed that NETs induce the iNOS-expressing M1 macrophage phenotype [53]. These antecedents leave evidence that the expression patterns of iNOS can vary according to various factors and extrapolation of the results should be avoided. In this work, iNOS expression in leukocytes stimulated in vitro with ETs, OVA or OVA-ETs in autologous leukocyte cultures was studied and it was used as a molecule for the profile of M1 macrophages, activated in the classical pathway [29]. As mentioned above, few studies have evaluated the potential effects of ETs on APCs, some were proinflammatory effects, and others anti-inflammatory [25]. In this research work, the results showed that although there was a higher percentage of cells marked with iNOS in the samples stimulated with OVA and ETs, unlike the controls, according to the statistical analysis there were no significant differences between the control samples and those stimulated with ETs, OVA or OVA and ETs. This allows us to infer that the components of autologous ETs did not behave as classical macrophage activation factors.

The results of this scientific research work on the influence of ETs, generated in vitro, on immune cells in autologous cultures of human blood allow us to provide data that contribute to the knowledge of multiple effects of their formation, in the context in which they were made the experimental trials. Influence of ETs on T cell activation was observed, and components of autologous ETs did not elicit classical activation of M1 macrophages.

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Conflict of Interest

Authors declare that they do not have any conflict of interest.

References

[1] Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil Extracellular Traps Kill Bacteria. *Science*

(80-). 2004; 303(5663): 1532-5.
[2] Muñoz-Caro T, Rubio R MC, Silva LMR, Magdowski G, Gärtner U, McNeilly TN, et al. Leucocyte-derived extracellular trap formation significantly contributes to Haemonchus contortus larval entrapment. *Parasit Vectors*. 2015; 8(1): 607.
[3] Germic N, Stojkov D, Oberson K, Yousefi S, Simon H-U. Neither eosinophils nor neutrophils require ATG5-dependent autophagy for extracellular DNA trap formation. *Immunology*. 2017; 152(3): 517-25.
[4] Papayannopoulos V, Zychlinsky A. NETs: a new strategy for using old weapons. *Trends Immunol*. 2009; 30(11): 513-21.
[5] Rocha Arrieta YC, Rojas M, Vasquez G, Lopez J. The Lymphocytes Stimulation Induced DNA Release, a Phenomenon Similar to NETosis. *Scand J Immunol*. 2017; 86(4): 229-38.
[6] Muniz VS, Baptista-dos-Reis R, Neves JS. Functional Extracellular Eosinophil Granules: A Bomb Caught in a Trap. *Int Arch Allergy Immunol*. 2013; 162(4): 276-82.
[7] Ueki S, Melo RCN, Ghiran I, Spencer LA, Dvorak AM, Weller PF. Eosinophil extracellular DNA trap cell death mediates lytic release of free secretion-competent eosinophil granules in humans. *Blood*. 2013; 121(11): 2074-83.
[8] Hamaguchi S, Seki M, Yamamoto N, Hirose T, Matsumoto N, Irisawa T, et al. Case of invasive nontypable Haemophilus influenzae respiratory tract infection with a large quantity of neutrophil extracellular traps in sputum. *Journal of Inflammation Research.* New Zealand; 2012: 137-40.
[9] Urban CF, Nett JE. Neutrophil extracellular traps in fungal infection. *Sem Cell Dev Biol*. 2018: 1-11.
[10] Agraz-Cibrian JM, Giraldo DM, Mary F-M, Urcuqui-Inchima S. Understanding the molecular mechanisms of NETs and their role in antiviral innate immunity. *Virus Res*. 2017; 228: 124-33.
[11] Díaz-Godínez C, Carrero JC. The state of art of neutrophil extracellular traps in protozoan and helminthic infections. *Biosci Rep*. 2019; 39(1).
[12] Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. *PLoS Pathog*. 2009; 5(10): e1000639.
[13] Vorobjeva N V, Pinegin B V. Neutrophil extracellular traps: mechanisms of formation and role in health and disease. *Biochemistry (Mosc)*. 2014; 79(12): 1286-96.
[14] Rodriguez FM, Novak ITC. Costimulatory Molecules CD80 and CD86 Colocalized in Neutrophil Extracellular Traps (NETs). *J Immunol Infect Dis*. 2016; 3(1): 1-9.
[15] Rodriguez FM, Clara Novak IT. May NETs Contain Costimulatory Molecules? *J Immunobiol*. 2016; 1(4): 113.
[16] Rinero R, Reyna M, Rodriguez F, Carabajal-Miotti C, Ruiz de Frattari S, Vargas A, et al. Beta Tubulin in Extracellular Traps and Mitochondrial Dynamics in Autologous Cultures of Human Leukocytes Stimulated With Lipopolysaccharide. *Clin Res Immunol*. 2018; 1(2): 1-5.
[17] Goldmann O, Medina E. The expanding world of extracellular traps: Not only neutrophils but much more. *Front Immunol*. 2012; 3: 1-11.
[18] Branzk N, Papayannopoulos V. Molecular mechanisms regulating NETosis in infection and disease. *Semin Immunopathol*. 2013; 35(4): 513-30.
[19] Kenny EF, Herzig A, Krüger R, Muth A, Mondal S, Thompson PR, et al. Diverse stimuli engage different neutrophil extracellular trap pathways. *Elife*. 2017; 6: 1-21.
[20] Berthelot J-M, Le Goff B, Neel A, Maugars Y, Hamidou M. NETosis: At the crossroads of rheumatoid arthritis, lupus, and vasculitis. *Jt bone Spine*. 2017; 84(3): 255-62.
[21] Sollberger G, Tilley DO, Zychlinsky A. Neutrophil Extracellular Traps: The Biology of Chromatin Externalization. *Dev Cell*. 2018; 44(5): 542-53.
[22] Borges L, Pithon-Curi TC, Curi R, Hatanaka E. COVID-19 and Neutrophils: The Relationship between Hyperinflammation and Neutrophil Extracellular Traps. *Mediators Inflamm*. 2020; 2020: 8829674.
[23] Wang J, Li Q, Yin Y, Zhang Y, Cao Y, Lin X, et al. Excessive Neutrophils and Neutrophil Extracellular Traps in COVID-19. *Front Immunol*. 2020; 11: 2063.
[24] Janiuk K, Jabłońska E, Garley M. Significance of NETs Formation in COVID-19. *Cells*. 2021; 10(1).
[25] Barrientos L, Bignon A, Gueguen C, de Chaisemartin L, Gorges R, Sandré C, et al. Neutrophil extracellular traps downregulate lipopolysaccharide-induced activation of monocyte-derived dendritic cells. *J Immunol*. 2014; 193(11): 5689-98.
[26] Donis-Maturano L, Sánchez-Torres LE, Cerbulo-Vázquez A, Chacón-Salinas R, García-Romo GS, Orozco-Uribe MC, et al. Prolonged exposure to neutrophil extracellular traps can induce mitochondrial damage in macrophages and dendritic cells. *Springerplus*. 2015; 4: 161.
[27] Kahlenberg JM, Carmona-Rivera C, Smith CK, Kaplan MJ. Neutrophil extracellular trap-associated protein activation of the NLRP3 inflammasome is enhanced in lupus macrophages. *J Immunol*. 2013; 190(3): 1217-26.
[28] Kumar V, Sharma A. Neutrophils: Cinderella of innate immune system. *Int Immunopharmacol*. 2010; 10(11): 1325-34.
[29] Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. 2013; 13(3): 159-75.
[30] Rodriguez FM, Novak ITC. What about the neutrophils phenotypes? *Hematol Med Oncol*. 2017; 2(3): 1-6.
[31] Tillack K, Breiden P, Martin R, Sospedra M. T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses. *J Immunol*. 2012; 188(7): 3150-9.
[32] Murphy K, Weaver C. *Janeway's Immunbiology*. 9th ed. Garland Science, editor. New York, USA: Taylor and Francis Group; 2017.
[33] Najmeh S, Cools-Lartigue J, Giannias B, Spicer J, Ferri LE. Simplified Human Neutrophil Extracellular Traps (NETs) Isolation and Handling. *J Vis Exp*. 2015; 98.
[34] Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open source platform for biological image analysis. *Nat Methods*. 2012; 9(7): 676-82.
[35] Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW. *InfoStat versión 2020*. Centro de Transferencia InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina; 2020.
[36] Tsai C-Y, Hsieh S-C, Liu C-W, Lu C-S, Wu C-H, Liao H-T, et al. Cross-Talk among Polymorphonuclear Neutrophils, Immune, and Non-Immune Cells via Released Cytokines, Granule Proteins, Microvesicles, and Neutrophil Extracellular Trap Formation: A Novel Concept of Biology and Pathobiology for Neutrophils. *Int J Mol Sci*. 2021; 22(6).
[37] Magna M, Pisetsky DS. The Alarmin Properties of DNA and DNA-associated Nuclear Proteins. *Clin Ther*. 2016; 38(5): 1029-41.
[38] Oberg H-H, Juricke M, Kabelitz D, Wesch D. Regulation of T cell activation by TLR ligands. *Eur J Cell Biol*. 2011; 90(6-7): 582-92.
[39] Lu H, Xu S, Liang X, Dai Y, Huang Z, Ren Y, et al. Advanced Glycated End Products Alter Neutrophil Effect on Regulation of CD(4)+ T Cell Differentiation Through Induction of Myeloperoxidase and Neutrophil Elastase Activities. *Inflammation*. 2019; 42(2): 559-71.
[40] Griffith ME, Coulthart A, Pusey CD. T cell responses to myeloperoxidase (MPO) and proteinase 3 (PR3) in patients with systemic vasculitis. *Clin Exp Immunol*. 1996; 103(2): 253-8.
[41] Fujiwara H, El Ouriaghli F, Grube M, Price DA, Rezvani K, Gostick E, et al. Identification and in vitro expansion of CD4+ and CD8+ T cells specific for human neutrophil elastase. *Blood*. 2004; 103(8): 3076-83.
[42] Tateosian NL, Reiteri RM, Amiano NO, Costa MJ, Villalonga X, Guerrieri D, et al. Neutrophil elastase treated dendritic cells promote the generation of CD4(+)FOXP3(+) regulatory T cells in vitro. *Cell Immunol*. 2011; 269(2): 128-34.
[43] Pulido R, Alvarez V, Mollinedo F, Sánchez-Madrid F. Biochemical and functional characterization of the leucocyte tyrosine phosphatase CD45 (CD45RO, 180 kD) from human neutrophils. In vivo upregulation of CD45RO plasma membrane expression on patients undergoing haemodialysis. *Clin Exp Immunol*. 1992; 87(2): 329-35.
[44] Cabral HRA, Novak ITC. Autologous rosette formation by human blood monocyte-derived macrophages and lymphocytes. *Am J Hematol*. 1999; 60(4): 285–8. doi: 10.1002/(SICI)1096-8652(199904)60:4<285::AID-AJH6>3.0.CO;2-A.
[45] Nikitina IY, Panteleev A V, Kosmiadi GA, Serdyuk Y V, Nenasheva TA, Nikolaev AA, et al. Th1, Th17, and Th1Th17 Lymphocytes during Tuberculosis: Th1 Lymphocytes Predominate and Appear as Low-Differentiated CXCR3(+)CCR6(+) Cells in the Blood and Highly Differentiated CXCR3(+/-)CCR6(-) Cells in the Lungs. *J Immunol*. 2018; 200(6): 2090-103.
[46] Petretto A, Bruschi M, Pratesi F, Croia C, Candiano G, Ghiggeri G, et al. Neutrophil extracellular traps (NET) induced by different stimuli: A comparative proteomic analysis. *PLoS One*. 2019; 14(7): e0218946.
[47] Lambert S, Hambro CA, Johnston A, Stuart PE, Tsoi LC, Nair RP, et al. Neutrophil Extracellular Traps Induce Human Th17 Cells: Effect of Psoriasis-Associated TRAF3IP2 Genotype. *J Invest Dermatol*. 2019; 139(6): 1245-53.
[48] Mariotti FR, Quatrini L, Munari E, Vacca P, Moretta L. Innate Lymphoid Cells: Expression of PD-1 and Other Checkpoints in Normal and Pathological Conditions. *Front Immunol*. 2019; 10: 910.
[49] Borregaard N. Neutrophils, from Marrow to Microbes. *Immunity*. 2010; 33(5): 657–70. doi: 10.1016/j.immuni.2010.11.011.
[50] Bogdan C. Nitric oxide synthase in innate and adaptive immunity: An update. *Trends Immunol*. 2015; 36(3): 161-78.
[51] Weinberg JB. Nitric oxide production and nitric oxide synthase type 2

expression by human mononuclear phagocytes: a review. *Mol Med*. 1998; 4(9): 557-91.

[52] Dijkstra G, Zandvoort AJH, Kobold ACM, de Jager-Krikken A, Heeringa P, van Goor H, et al. Increased expression of inducible nitric oxide synthase in circulating monocytes from patients with active inflammatory bowel disease. *Scand J Gastroenterol*. 2002; 37(5): 546-54.

[53] Josefs T, Barrett TJ, Brown EJ, Quezada A, Wu X, Voisin M, et al. Neutrophil extracellular traps promote macrophage inflammation and impair atherosclerosis resolution in diabetic mice. *JCI insight*. 2020; 5(7).