Human Polymorphonuclear Neutrophil Phenotypes Generated in vitro

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ABSTRACT

**Background:** There are a variety of polymorphonuclear neutrophil phenotypes described in different species and health or disease situations.

**Objective:** Study human neutrophil phenotypes generated in vitro. Methods: Heparinized human blood samples were collected with ethical consent. Polymorphonuclear neutrophils purification and autologous cultures was performed. Neutrophil stimulation was performed with LPS, fMLP or OVA. Immunofluorescence was applied.

**Results:** “Polymorphonuclear neutrophil-antigen presenting cell” profile was generated in vitro, expressing CD80, CD86 and HLA-DR molecules. Immunofluorescence analysis showed: CD80 expression, significant differences between CTFT control and CTFT fMLP (p<0.05), CTFT control and CTFT OVA (p<0.0001). CD86 expression, significant differences between CTFT control and CTFT fMLP (p<0.05), CTFT control and CTFT LPS (p<0.05), CTFT control and CTFT OVA (p<0.0001). HLA-DR expression, significant differences between CTFT control and CTFT LPS (p<0.05). About “Polymorphonuclear neutrophil-C4D-C4D5RO” profile, analysis show: CD4 expression, significant differences between CTFT control and CTFT fMLP (p<0.05), CD45RO expression, no significant differences. "Polymorphonuclear neutrophil-APC" phenotype, released NETs expressing CD4 and C45RO expression, no significant differences. “Polymorphonuclear neutrophil-antigen presenting cell” phenotype, released NETs with CD80, CD86 at 30 minutes: paired control samples (7.4%), stimulated with LPS (12.69%), fMLP (16.67%) and OVA (18.47%). HLA-DR expression in NETs, at 30 minutes, in paired control samples (0%), stimulated with LPS (16.17%). At 17 hs, in paired control samples (0%), with OVA stimulation (4.54%). “Polymorphonuclear neutrophil-C4D-C4D5RO” phenotype, released NETs expressing CD4 and C45RO molecules. At 30 minutes, in paired control samples (0%), stimulated with LPS (7.67%), fMLP (6.38%) and OVA (0%).

**Conclusions:** Molecules expressed by phenotypes can play a relevant role by influencing cellular microenvironment and can be taken into account as possible therapeutic targets.

**Keywords:** Human neutrophil, NETs, polymorphonuclear neutrophil phenotypes.

I. INTRODUCTION

Polymorphonuclear neutrophils (PMN) are currently considered cells that actively participate in adaptive immunity. The functionalities of this leukocyte include neutrophil extracellular traps or NETs [1] and potential ability to behave as an antigen-presenting cell (APC) [2]. It is necessary to name existence of multiple phenotypes in different species and health or disease situations [3],[4].

In relation to the multiplicity of roles in immune responses, PMN neutrophil plasmatic membrane has various receptors [5] which allows it to perform versatile possible interactions.

PMN neutrophils perform innate immune recognition through Toll-like receptors against bacterial infections, for example TLR4 (plus MD-2 and CD14), which binds lipopolysaccharide (LPS) from Gram-negative bacteria and lipoteichoic acids from Gram-positive bacteria.

Among neutrophil marker molecules, CD66 molecule stands out [6], this molecule is found as CD66a or CD66b in positive bacteria and negative bacteria and mannans. On the other hand, by
binding to CD66a and CD66b, it causes grouping of receptors, promoting adherence and improving phagocytic capacity [8].

PMN neutrophils also express the costimulatory molecule CD40 on their plasma membrane [9] and this allows them to interact with CD40L-bearing T cells and plasmacytoid dendritic cells. Through CD40-CD40L pathway, two-way signaling is induced that transmits activating signals to T cells and also induces B7 molecules expression in cell that carries CD40 [10].

The expression of CD80, CD86 and the Major Histocompatibility Complex Class II (HLA-DR) molecules in physiological and pathological conditions, characterize the PMN neutrophil phenotype as APC [2].

B7 molecules are integral membrane glycoproteins expressed on professional APCs. In monocytes, macrophages and dendritic cells without stimulation, B7-1:CD80 molecule expression is absent or low. B7-2:CD86 molecule on the same cells is constitutively expressed [11]. These B7 molecules are produced on APCs in response to signaling by stimulation with pathogen-associated molecular patterns (PAMPs) such as LPS through the engagement of TLR4 [10]. Substances such as LPS that induce costimulatory activity have been used for years as "adjuvants" to induce immunogenicity in protein antigens by administering them together in vaccines [10].

HLA-DR molecule of Major Histocompatibility Complex Class II (MHC II) is involved in processing and presentation of antigens via class II or endosomal pathway. It has been shown that this molecule is not only expressed in professional antigen presenting cells such as macrophages, dendritic cells and B lymphocytes. Its expression has also been reported together with costimulatory molecules B7 (CD80 and CD86) in PMN neutrophils, molecules required for antigen presentation and T cell activation, under action of certain stimuli such as phorbol myristate acetate, N-formyl methionyl leucyl phenylalanine (fMLP), LPS, phagocytosis of immunoglobulin G-Latex particles [12] and the crosslinking of Mac-1 (CD18 + CD11b) [13]. In works of this author and his collaborators, it is reflected that costimulatory molecule B7-1:CD80 is found preformed in secretory vesicles together with HLADR (MHC II) and costimulatory molecule B7-2:CD86 in secretory vesicles, secondary and azurophilic granules [12],[13].

Regarding the topic of PMN neutrophil as APC, it has been seen that several proinflammatory cytokines produced at inflammation sites activate PMN neutrophils, suppress their apoptosis, and these cytokine-activated PMN neutrophils show expression of molecules that make them competent for antigen presentation [14]. In certain diseases, PMN neutrophils take on APC’s characteristics, for example in patients with Wegener's granulomatosis [15]. PMN neutrophils isolated from synovial fluid of rheumatoid arthritis patients have also been reported to express MHC Class II molecules [16]. Differentiation of PMN neutrophils into a hybrid cell population was experimentally described in murine models, exhibiting a dual phenotype with PMN neutrophils and dendritic cells functions [17]. Presentation of antigens restricted to Class II in murine models has also been reported [18],[19]. In other experimental situations, murine PMN neutrophils can behave as APCs capable to differentiate lymphocyte into Th1 and Th17 effector cells [20].

On the other hand, unconventional molecules expression in PMN neutrophils, such as CD4 and CD45RO, has been reported, although there is very little bibliography on the matter [21],[22].

CD4 molecule originally identified as a T helper lymphocyte marker, is co-receptor of T helper CD4 lymphocyte, which binds Class II molecules at antigen presentation time. It can also be expressed on monocytes, macrophages, eosinophils [23], CD34+ progenitor cells [24],[25], CD8+ cells infected in vitro by human herpes virus 6 (HHV-6 )[26], NK cells[27], mast cells, basophils [28] and gamma/delta T cells [29]. Biswas et al, demonstrated in their results that a low percentage of healthy people infected with HIV had CD4 (+) PMN neutrophils and percentage of these CD4 (+) neutrophils was highly variable (between 39 and 97% of total PMN neutrophils) [21].

Regarding CD45 molecule, it is considered an activation marker, with tyrosine phosphatase activity expressed in all hematopoietic nucleated cells and their precursors, except red blood cells and platelets [30]. In T cells, CD45 molecule dephosphorylates Lyck tyrosine kinase as part of the TCR activation signaling cascade [10]. CD45RO molecule is an activation state marker in effector and memory T cells [10],[31], but has also been observed in some PMN neutrophils phenotypes from hemodialysis patients and in vitro activated PMN neutrophils with fMLP formylated peptides [22],[32]. This molecule is expressed in PMN from healthy patients but no natural ligand has been found [32]. CD45RO localizes to PMN-specific granules [22]. It is known that CD45RO molecule isoform is expressed when T cell is activated, and it will associate with TCR and co-receptor (CD4 or CD8), making T cell more sensitive to stimulation by low concentrations of peptide-antigen complexes. When cells differentiate into effectors and express CD45RO isoform on the surface, it lacks A exon present in CD45RA isoform that is expressed on naïve cells surface [10]. Thus, depending on the maturation state, activation, and differentiation, T cells express various isoforms of CD45: CD45RA, CD45RB, or CD45RO.

The arsenal of functional mechanisms of PMN neutrophils includes phagocytosis, degranulation, reactive oxygen species (ROS) synthesis, and NET generation. The latter are structures formed by chromatin, histones and granular proteins that are released into extracellular matrix in sterile and non-sterile inflammatory conditions, according to Brinkmann et al 2004 [1],[33]. Since there are numerous stimuli that can release them and are involved in defense, as well as in causing pathological phenomena, it is interesting to study their structure and functions [34],[35].

In a previous work we described CD80 and CD86 molecules expression in NETs, B7 costimulatory molecules that intervene in one of the most studied costimulation pathways -the B7-1 B7-2 CD28 CTLA-4 pathway- this finding opens a window to explain new immunoregulation mechanisms and peripheral self-tolerance breakdown, since once released, these molecules could influence responses in the microenvironment where they are found, according to different subpopulations of naive, memory, effector and regulatory T lymphocytes that are compromised [36].
In the present work we demonstrate the expression of molecules that characterize the PMN neutrophil as APC in purified neutrophils, as well as a PMN neutrophil profile that is capable to express CD4 and CD45RO molecules. Interestingly, we also report the finding of HLA-DR expression and unconventional molecules such as CD4 and CD45RO in NETs.

II. MATERIAL AND METHODS

A. Samples

Heparinized human blood samples (n = 50) were collected with ethical consent according to procedures approved by ethical committee of National Hospital Clinicas R169/13, I minute book N129. Samples donated by the Blood Bank, Institute of Hematology and Hemo therapy of the National University of Cordoba in anonymity, with negative serology: Hudleson (Wiener), VDRL (Wiener), Chagras HAI (Wiener) Chagas EIE (Biomerieux), HBs EIE (Biomerieux), HBe (Biomerieux), HCV EIE (Murex), HIV Ac EIE (Biomerieux), HIV Ag EIE (Biomerieux), HTLV EIE (Murex).

B. PMN Neutrophils Purification

PMN neutrophils purification was performed using Histopaque ® Gradient 1119 and 1077 according to the manufacturer's data sheet (Sigma).

C. Autologous Cultures of Purified PMN Neutrophils

Blood samples obtained by the method already described above were used to culture purified neutrophils 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.

D. PMN neutrophils stimulation

Neutrophils stimulation was performed by adding different stimulators to the wells of culture plates containing total leukocytes or purified PMN neutrophils with culture medium at time zero. In all cases there were samples of control cultures without the addition of stimulators.

Stimulation with lipopolysaccharide LPS (Sigma Aldrich). LPS was added at a concentration of 25 ng/ml. Samples were taken at different times, depending on the experimental situation.

Stimulation with fMLP (phenyl-Met-Leu-Phe) formylated peptides (Sigma Aldrich). fMLP was added at a concentration of 0.25 ng/ml. Samples were taken at 30 minutes.

Ovalbumin (OVA) stimulation. A final concentration of 100 µg/ml was used. Samples were taken from cultures at different times according to the experimental situation.

E. NETs Generation

For the generation of NETs, the previously mentioned classical (LPS, fMLP) and non-classical (OVA) PMN neutrophils stimulators were used. Cultures were sampled at different times, depending on the experimental situation, to observe the occurrence of NETs. The released NETs were visualized by fluorescence microscopy using DAPI (4,6'-diamino-2-phenylindole) (Sigma, St Louis, MO) for DNA staining.

F. NETs Quantification

The released NETs were visualized by immunofluorescence microscopy and the percentage of PMN neutrophils releasing NETs with CD80, CD86, CD4, CD45RO, HLA-DR colocalized in NETs was calculated as the mean value across four fields (1000x) normalized by the total number of cells.

G. Samples for Immunofluorescence

Glass cover slips with attached cells were carefully removed from culture plate and immunofluorescence techniques were performed.

H. Immunofluorescence (IF)

Culture cells 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) Santa Cruz Biotechnology anti-CD80 (FITC; Santa Cruz Biotechnology), anti-CD86 (PE; Santa Cruz Biotechnology), anti-HLA DR (FITC; Santa Cruz Biotechnology) antibodies; anti-CD45RO (FITC; Santa Cruz Biotechnology) and anti-CD4 (PE; 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.

I. Quantitative Relative Immunofluorescence Analysis of Images

All quantifications were performed with the FIJI-ImageJ image processing software (National Institutes Of Health, Bethesda, MD, USA) [37], [38]. A mask was created for each image to be analyzed. The regions of interest (ROI) were obtained from said mask and the relative fluorescence measurements were made through the “Analyze particles” command. The following parameters were taken into account: Area of each cell according to ROI, Integrated Density (IntDen) that measures the general fluorescence intensity of each ROI, Mean background (MB) calculated as average signal for a selected region right next to the cell, Corrected total cell fluorescence (CTFT) was obtained according to the following formula CTFT = IntDen - (Cell area x MB) [39],[40]. IntDen and CTFT were expressed in relative fluorescence units (RFU).

J. Statistical Analysis

In NETs quantification, percentage of positive cells releasing NETs was calculated as the mean value in four fields normalized by the total number of cells. Data were expressed as mean value ±SD. They represented three independent experiments. Data were analyzed with Student's t-test for paired samples using InfoStat software [41], p<0.05 was considered statistically significant.
In the quantitative analysis of relative fluorescence of the images, the average of IntDen and CTFT per sample was calculated. Data were expressed as mean value ± SD. Statistical analysis of mean CTFT values was performed by Student's t-test for paired samples using InfoStat software, p<0.05 was considered statistically significant.

III. RESULTS

A. Expression of CD80, CD86 and HLA-DR in purified PMN Neutrophil Autologous Cultures in vitro

![Image of CD80, CD86, HLA-DR expression](image1)

The phenotype that expresses costimulatory molecules B7 and HLA-DR was generated in vitro, which we will call “PMN neutrophil-APC” (Fig. 1 and Fig. 2). In the analysis of the IF images with the FIJI-ImageJ software, the results detailed below were obtained. For CD80 expression: CTFT control 1,172,594.83 ± 304,923.25 RFU, CTFT fMLP 1,608,528.15 ± 643,131.73 RFU, CTFT LPS 1,378,431.20 ± 776,553.97 RFU, CTFT OVA 682,069.01 ± 258,961 RFU, with significant differences between CTFT control and CTFT fMLP (p<0.05), CTFT control and CTFT OVA (p<0.0001) (Fig. 1E). For CD86 expression: CTFT control 482,256.2 ± 167,384.71 RFU, CTFT fMLP 696,800.15 ± 240,044.99 RFU, CTFT LPS 893,929.61 ± 649,584.27 RFU, CTFT OVA 255,178.93 ± 114,051.82 RFU, with significant differences between CTFT control and CTFT fMLP (p<0.05), CTFT control and CTFT LPS (p<0.05), CTFT control and CTFT OVA (p<0.0001) (Fig. 1F). For HLA-DR expression: CTFT control 394,415.04 ± 190,594.18 RFU, CTFT LPS 768,112.16 ± 307,133.93 RFU, with significant differences between CTFT control and CTFT LPS (p<0.05) (Fig. 2C).

B. Expression of CD4 and CD45RO in purified PMN neutrophils autologous cultures in vitro

![Image of CD4, CD45RO expression](image2)

![Image of HLA-DR expression](image3)

On the other hand, the phenotype that expresses the CD4 and CD45RO molecules was generated in vitro, which we will call “PMN neutrophil-CD4-CD45RO” (Fig. 3). In the analysis of the IF images with the FIJI-ImageJ software, the following results were obtained. For CD4 expression: CTFT control 389,833.09 ± 422,815.42 RFU, CTFT fMLP 1,019,227.84 ± 274,374.28 RFU, CTFT LPS 341,425.77 ± 102,784.90 RFU, CTFT OVA 234,387.93 ± 117,313.93 RFU, with significant differences between CTFT control and CTFT fMLP (p<0.05) (Fig. 3E). For CD45RO expression: CTFT control 389,833.09 ± 422,815.42 RFU, CTFT fMLP 1,019,227.84 ± 274,374.28 RFU, CTFT LPS 341,425.77 ± 102,784.90 RFU, CTFT OVA 234,387.93 ± 117,313.93 RFU, with significant differences between CTFT control and CTFT fMLP (p<0.05) (Fig. 3E). For CD45RO expression: CTFT control 389,833.09 ± 422,815.42 RFU, CTFT fMLP 1,019,227.84 ± 274,374.28 RFU, CTFT LPS 341,425.77 ± 102,784.90 RFU, CTFT OVA 234,387.93 ± 117,313.93 RFU, with significant differences between CTFT control and CTFT fMLP (p<0.05) (Fig. 3E). For CD45RO expression: CTFT control 389,833.09 ± 422,815.42 RFU, CTFT fMLP 1,019,227.84 ± 274,374.28 RFU, CTFT LPS 341,425.77 ± 102,784.90 RFU, CTFT OVA 234,387.93 ± 117,313.93 RFU, with significant differences between CTFT control and CTFT fMLP (p<0.05) (Fig. 3E). For CD45RO expression: CTFT control 389,833.09 ± 422,815.42 RFU, CTFT fMLP 1,019,227.84 ± 274,374.28 RFU, CTFT LPS 341,425.77 ± 102,784.90 RFU, CTFT OVA 234,387.93 ± 117,313.93 RFU, with significant differences between CTFT control and CTFT fMLP (p<0.05) (Fig. 3E). For CD45RO expression: CTFT control 389,833.09 ± 422,815.42 RFU, CTFT fMLP 1,019,227.84 ± 274,374.28 RFU, CTFT LPS 341,425.77 ± 102,784.90 RFU, CTFT OVA 234,387.93 ± 117,313.93 RFU, with significant differences between CTFT control and CTFT fMLP (p<0.05) (Fig. 3E).
RFU, without significant differences between control CTFT and CTFT of the other trials (Fig. 3F).

C. Expression of CD80, CD86 in NETs of Purified Autologous PMN Neutrophils Cultures in vitro

The generation of NETs was observed in the PMN neutrophil-APC phenotype, expressing costimulatory molecules B7: CD80, CD86 in the NETs (Fig. 4). At 30 minutes, in paired control samples without stimulation, the formation of NETs was observed in 7.4% of the cells expressing CD80 and CD86. After stimulation with LPS, fMLP and OVA, the percentage of cells that released NETs expressing CD80 and CD86 was recorded with values of 12.69%, 16.67% and 18.47%, respectively (Fig. 4).

D. HLA-DR Expression in NETs of Purified Autologous PMN Neutrophils Cultures in vitro

On the other hand, the generation of NETs was also observed in the PMN neutrophil-CD4-CD45RO phenotype, expressing the CD4 and C45RO molecules in the NETs (Fig. 6). At 30 minutes, in paired control samples without stimulation, the formation of NETs expressing CD4 and CD45RO was not observed. After stimulation with LPS and fMLP, the recorded percentage of cells that released NETs expressing CD4 and CD45RO was 7.67% and 6.38%, respectively. In samples stimulated with OVA, NETs with CD4 and CD45RO were not observed (Fig. 6E).

IV. DISCUSSION

Neutrophil PMNs have classically been characterized as typical cell of acute inflammation marking the first line of defense in fungal and bacterial infections, but there is now evidence that they are also actively involved in adaptive immunity [42]. It is also known that an imbalance in favor of its hyperactivity results in tissue damage, as occurs in severe inflammation and trauma [43].

In recent years, NETs study has focused on involvement of pathophysiogenic phenomena of diseases [44] such as rheumatoid arthritis, systemic lupus erythematosus,
Alzheimer's disease, cystic fibrosis, thrombotic diseases, cancer, sepsis, among others [34]. Various studies have shown that NETs constituent proteins trigger diseases according to their mechanism of action [45, 46], for example enzymes that promote thrombosis [47, 48], persistent tissue damage [49] and on the other hand, these proteins have also been shown to be sources of autoantigens triggering autoimmune phenomena [50, 51]. On the other hand, CD80 and CD86 costimulatory molecules colocalized in NETs could also contribute to self-tolerance breakdown or regulation of immune response, through B7-1/B7-2 pathway: CD28/CTLA-4 [36].

Another issue, still under study, is characterization of different PMN neutrophils phenotypes, since it has been observed they can express different molecules, under different stimuli, in different species, either under physiological and/or pathological conditions [52]. Relatively recently, two new neutrophil profiles have been described, one induced by IL-23 [53] and the other induced by IL-33 [54], which could reveal a broader polarization apart of already defined N1/N2 classification [55].

This plasticity of human PMN neutrophils allows them to behave like APCs by expressing on surface molecules required for antigen presentation and T cell activation, such as MHC Class II (HLA-DR), in addition to costimulatory molecules B7: CD80 and CD86 [2]. Marker molecules expression of "PMN neutrophil-APC" phenotype after stimulation with LPS, fMLP and OVA in purified PMN neutrophil cultures is corroborated in this research work. Recently, a study with human PMN neutrophils has been published where acquisition of ability to present antigens to memory CD4 T cells is described, by expressing HLA-DR (MHC II) and costimulatory molecules B7 [56]. In this publication it is clarified that innate stimuli only achieved co-stimulatory molecules expression and did not provoke MHC II expression in contrast to our results. They only achieved HLA-DR (MHC II) expression with the requirement of specific antigen and specific memory CD4 T cells [56]. Interestingly, presence of MHC II-expressing neutrophil PMNs in unstimulated lymph nodes has been reported, and ex vivo stimulation with IgG-OVA immunocomplexes also results in MHC II expression in circulating neutrophils [57].

On the other hand, through the tests carried out in this work, “PMN neutrophil-CD4-CD45RO” phenotype was generated in vitro by stimulating purified PMN neutrophils with LPS, fMLP and OVA. It has been strikingly observed that peripheral blood PMN neutrophils can unconventionally express the CD4 molecule in a superficial or cytoplasmic form, in healthy patients and in HIV-positive patients [21]. As is known, CD4 is co-receptor for helper T cells and primary receptor for HIV virus [21]. CD45-RO molecule, a characteristic marker of memory and effector T cells, is a tyrosine phosphatase that regulates lymphocyte activation [22] and its expression in PMN neutrophils has been described in 96.7% ± 2.6 % on cell surface [32]. CD45 has also been reported to modulate respiratory burst activation and its engagement synergizes with formylated peptide stimulation of respiratory burst [58]. In addition, PMN neutrophil chemotactic response is affected when CD45 epitopes interact with leukotriene B4 and Complement C5a receptor-associated molecules [59]. CD45 can also modulate PMN antibody-dependent cytotoxicity, increase IL-6 production through FcyRIIa [60] and suppress protein tyrosine kinase p56ck expression of Src family [32]. In interaction with cytoskeleton components, CD45RO is involved in adhesion processes regulation [61].

As mentioned, NETs generation is a functional mechanism in constant exploration, so another objective was to demonstrate that characterized phenotypes molecules are expressed not only on PMN neutrophil surface, but also into NETs. In this work, HLA-DR molecule expression is demonstrated, in addition to costimulatory molecules B7 expression and CD4 and CD45RO unconventional molecules in NETs. Our assumption that it was feasible to find this set of molecules in NETs related to described PMN neutrophils phenotypes (PMN neutrophil-APC and PMN neutrophil-CD4-CD45RO) is based on bibliography that supports these molecules are contained in cytoplasmic reservoirs [12, 13, 21, 22]. Let us remember NETs are constituted of granular components and their composition can vary depending on the stimulus [62]. PMA, LPS, cytokines, an others, are some stimuli which can release NETs. It is known spontaneous NETs formation is also feasible, in our tests on paired samples without stimulation this was visible in coincidence with bibliography [63], in these cases NETs generation may have occurred as a consequence of neutrophils spontaneous activation due to their handling or temperature variations, as well as it has been documented autologous cultures of purified PMN, free of donor serum, NETs release is favored [64].

Regarding OVA use as a non-classical activator NETs inducer, the presence of antigen carried by neutrophils in lymphoid organs of OVA immunized mice was determined in murine experiments performed by other researchers [65]. Although it has been generally accepted neutrophils are not involved in adaptive responses, OVA antigen uptake by neutrophils was observed in those experiments. These neutrophils mainly secrete TNF-α and authors conclude this would allow them to participate as immunomodulatory cells [65]. In lymph nodes, CD4 (+) T cells population expands after PMN neutrophils arrival, but on the other hand, PD-L1 molecules are upregulated with consequent suppression of CD4 (+) T cell proliferation [66]. NETs can be induced by OVA:anti-OVA immune complexes [63] and in other granulocytes, OVA-induced extracellular trap (ET) formation was described [67]. Also due to our previous experience in previous trials, where it was suggested OVA assays results would probably correspond to stimulation by OVA alone or by stimulation with OVA-anti-OVA immune complexes, due to the possible existence of anti-specific OVA in donor serum, it was decided to include this activator also in this investigation. After the challenge with LPS, HLA-DR expression was not evidenced at 30 minutes, but it was observed hours later. The reason for this pattern of expression should be asked, which will require further study. It is known that when MHC molecules are on cell surface, some binding with extracellular peptides can occur, although it is not clear whether this phenomenon is due to presence of empty MHC molecules or to peptide exchange. However, it can happen and is a widely used technique for loading synthetic peptides to test for T-cell specificity [10]. Classically, it has been described antigenic peptide is generated intracellularly and
binds stably to antigen-binding site on MHC molecule surface. Addition of peptides to living or even chemically fixed cells in vitro can generate antigenic peptide-MHC complexes that are recognized by peptide-specific T cells [10]. The finding of MHC II (HLA-DR) molecules in NETs may imply the possibility of antigenic presentation.

On the other hand, presence of B7 co-stimulatory molecules in NETs is interesting because it would explain diseases pathophysiology in which these molecules could be involved [68]. Unlike our previous work carried out in total leukocytes cultures [36], present study was performed in purified PMN neutrophils, a higher percentage of PMN neutrophils that release NETs containing CD80 and CD86 when stimulated with OVA is observed with respect to control, but even so, the percentage is lower than that found in whole blood cultures. This may indicate presence of other leukocytes, or cytokines released by these leukocytes, or interactions between different cell types contribute to best performance in terms NETs releases by PMN neutrophils, thus highlighting the importance make experimental conditions as close to physiological as possible. Interestingly, existence of costimulatory B7 molecules soluble forms must be added: CD80 (sCD80), CD86 (sCD86), also CTLA-4 (sCTLA-4) [69]–[72] and HLA-DR (sHLA-DR) [73], [74] that are functionally identical to their membrane pairs, which could explain why B7 molecules released by NETs can also exert its costimulation functions. On the other hand, phenomenon called trogocytosis (from the Greek), consist in membrane fragments or surface molecules transfer from one cell to another while retaining its functional capacity [75], could also occur if molecules are released by NETs. That is, B7 CD80 and CD86 molecules released by NETs could play their costimulation role, and trigger complete activation, inhibition or anergy in other cells. This could happen, for example, in autoimmune diseases such as RA, Sjögren's disease, SLE, among others, where NETs occurrence is described as a pathophysiological mechanism [34], [45], [76]. The finding of CD4 and CD45R0 molecules in NETs is important for future studies where functional relevance of these molecules in a given environment can be challenged. Medical importance regarding CD4 molecule in PMN neutrophils is related to possibility to influence HIV biodistribution [21]. Interestingly, CD4 soluble form (sCD4) has been reported, it would inhibit HIV entry into cells [77]. About CD45R0 molecule, it can trigger multiple actions in PMN neutrophils or microenvironment where it is found, with respect to chemotaxis, phagocytosis, among others mentioned above, these interactions could be taken into account for development of CD45 regulatory agents, constituting a new therapeutic approach for treatment of inflammatory diseases [78].

The different PMN neutrophils phenotypes could play an important role in immunomodulation, it is recognized that these leukocytes are extremely interesting cells, capable of modulating innate and adaptive immune responses in its activation and regulation according to their microenvironment and interaction with other cell types [79]. The variability of their functions according to their environment, physiological or pathological condition, and expression of different surface markers have made it possible to characterize different neutrophil profiles in murine and human models [3]. Currently, many described PMN neutrophils phenotypes are considered [2]–[4], [80], but such descriptions in bibliography are heterogeneous since parameters, methods, species, tissues and biomarker molecules are different. However, all studies are valid in the context developed with care to avoid extrapolation.

In short, results obtained are important, since they can be taken into account as possible therapeutic targets for diseases treatment where molecules expressed by phenotypes can play a relevant role by influencing cellular microenvironment where they are found. Future research on impact of NETs that express studied phenotypes molecules, on T cells role or other immune cell types, would be of great interest.

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CONFLICT OF INTEREST

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

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