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Influence of B7 costimulatory molecules colocalized in Extracellular Traps (ETs) on the expression of CD45RO in neutrophils and CD4 lymphocytes in human autologous cultures

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Abstract
Extracellular traps (ETs) are structures composed of chromatin, histones and granular proteins, which constitute a functional microbicidal mechanism of immune cells. Currently, its participation in the pathophysiology of various pathologies, including COVID-19, autoimmune diseases and cancer, has been studied. Costimulatory B7 molecules (B7-1:CD80 and B7-2:CD86) provided the second activation signal for T cells. In this research, the study of the influence of colocalized B7 molecules in ETs was carried out *in vitro* from leukocytes on T-cell activation and CD45 RO expression in neutrophils in autologous human blood cell cultures. Objectives: to generate and isolate ETs in autologous cultures of leukocytes exposed to lipopolysaccharide (LPS); labelling B7 molecules in isolated ETs, CD4 co-receptor expression and CD45RO activation marker in interaction assays with ETs and autologous leukocytes in culture. Methods: autologous leukocytes cultures from healthy human blood samples anticoagulated with heparin, (n = 10) with informed consent (Ethics Committee, HNC, FCM), were stimulated with 25 ng/ml of LPS, 30 minutes. Subsequent isolation technique. Immunofluorescence technique with anti-CD80, anti-CD86, anti- CD4 and anti-CD45RO was performed, DNA staining: DAPI. Controls: paired blood samples. Statistical treatment: *t*-test for paired samples. Results: At 24 hours of culture, no significant differences ($p<0.05$) were observed in percentages of CD4 and CD45RO positive cells between paired control samples and ETs addition samples. At 72 hours of culture, significant differences ($p<0.05$) were observed in CD45RO expression between controls and ETs addition samples. In one donor, significant differences ($p<0.05$) were observed in CD4-positive cells percentage. The presence of B7 molecules could give the second signal required for the activation of autoreactive T lymphocytes present in the autologous culture, implying the possibility of breaking self-tolerance.

Keywords: Extracellular traps, costimulatory B7 molecules, TCD4 cells, CD45RO

Introduction
Extracellular traps (ETs) are structures composed of chromatin, histones, and granular proteins, which were initially described in polymorphonuclear neutrophils (PMNs) leukocytes by Brinkmann *et al.* [1] calling them "neutrophil extracellular traps" (NETs). Chromatin content in NETs has led to the postulation of Immunity as its second function [2]. In this sense, DNA would fulfill a function that extends beyond RNA and protein sequences coding, since it also serves to trap bacteria and other extracellular pathogens, and functions as a valuable scaffold for antimicrobial mediators such as the granule proteins of the immune cells [3-5]. NETs components are still under study and approximately 30 constituent proteins have been described [6, 7]. In our laboratory, findings of particular interest have been made for this topic, since the presence of costimulatory molecules B7 CD80 and CD86 in NETS was described [8, 9], as well as the presence of beta-tubulin [10]. NETs formation (NETosis) can be generated by various pathogens such as bacteria, fungi, protozoa, viruses, as well as components of bacterial cell wall such as lipopolysaccharides (LPS) [1, 5, 7].

NETosis can also be induced by antibodies and immune complexes, cytokines and chemokines (IL-8, TNF), microcrystals, and other physiological stimuli [7].

Conventional suicidal NETosis initiates with binding of ligands to neutrophil Toll-like receptors and receptors for IgG-Fc, C, or cytokines [1]. Upon activation of these receptors, calcium stores in endoplasmic reticulum (ER) release calcium ions into the cytoplasm. Elevated cytoplasmic calcium levels increase protein kinase C (PKC) activity and gp91phox [11]. This induces the assembly of the cytosolic and membrane-bound subunits of NADPH oxidase into functional complexes on cytoplasmic or phagosomal membranes (also called phagocytic oxidase, PHOX) and the subsequent generation of ROS [12]. Under ROS influence, granules and nuclear envelope rupture. Then, the nuclear, granular and cytoplasmic contents are released. Neutrophil elastase (NE) and myeloperoxidase (MPO) enzymes, usually stored in azurophilic granules, migrate to the nucleus. Here, NE degrades histone H1 linker and processes core histones, MPO facilitates chromatin decondensation [12]. Histone removal by peptidyl arginine deaminase 4 (PAD4) and proteolytic histone cleavage initiated before nuclear cleavage contribute to chromatin decondensation [12]. In this way, nuclear, granular and cytoplasmic components are mixed. Later, plasma membrane rupture occurs, allowing NETs to release and leading to loss of viable cellular functions such as migration, phagocytosis, and cell death. On the other hand, in recent years the so-called vital NETosis existence has been described where nucleus chromatin is released by vesicles that fuse with the external membrane, which allows NETs to release of through these vesicles into the extracellular space, while the plasma membrane remains intact [13].

Regarding NETs and their relationship with diseases, it is important to note that physiological processes occurring in the organism must be controlled by strict mechanisms. Without regulation, any physiological process can have serious pathological consequences. NETs are no exception. Although physiological amounts of NETs are important as anti-infective agents in innate immune response, aberrant high levels of NETs in circulation can result in opposite pathological conditions generating, for example, microthrombi in capillaries, impaired microcirculation and tissue damage [14].

In certain patients with autoimmune diseases such as systemic lupus erythematosus (SLE) or systemic vasculitis, large amounts of autoantibodies against double-stranded DNA (dcDNA), against histones, and against MPO are produced. Since these molecules are abundant in NETs, it could have a role in autoimmune diseases development [12]. It has been suggested in SLE patients, autoantibodies would activate neutrophils promoting NETs release. These NETs would activate plasmacytoid dendritic cells and lead to interferon-α release, exacerbation or perpetuation of inflammation and infection [15]. It was possible to demonstrate that certain patients with SLE show a slow degradation of NETs *in vitro*, possibly due to the presence of certain self-DNase inhibitors or antibodies that protect NETs. Those patients who present an alteration in NETs degradation develop lupus nephritis [16].

In addition, NETs formation with sterile inflammatory stimuli from monosodium urate crystals has been described, which is important in patients suffering from gout [17]. NETs contribute to autoimmune pathologies development since they are potential sources of antigens [18].

In the COVID-19 pandemic scenario, NETs influence on pathology has been observed and studied. Veras *et al.* investigated the potentially detrimental role of NETs in the pathophysiology of 32 hospitalized patients with severe COVID-19, they found NET levels increased in tracheal aspirate and plasma, in addition to their neutrophils naturally produced significant NETs concentrations [19]. In virus infection, neutrophils are effective in blocking viruses at infection site, entrapping them in a DNA lattice. Virus-induced NETosis process, in this case, 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 NETs release that cause damage to organism. Wang *et al*. showed that several patients with COVID-19 had rising neutrophil counts and falling lymphocyte counts during severe phase of SARS-CoV-2 infection [20]. Similarly, Barnes *et al*. 2020, observed extensive neutrophil infiltration in the pulmonary capillaries of a patient with COVID-19 [21]. NET overproduction would induce lung tissue damage by NETosis-related enzymes such as NE and MPO. Hence, it is valid to analyze and explain, expanding the studies, the relationship between excessive amount of neutrophils and ETs overproduction in COVID-19 infection symptoms and the relationship between hyperinflammation (overproduction of NETs and storm of cytokines) and neutrophils role in destroying the viral infection [22].

On the other hand, it has been studied that NETs favour micro thrombosis, contribute to cancer cell metastasis progress, and produce permanent damage in pulmonary, cardiovascular, and renal systems [23].

Regarding activation of T lymphocytes and costimulatory molecules: B7-1 and B7-2, an adequate adaptive response by lymphocytes depends on the tight regulation of lymphocyte activation signals. This requires two signals, the first signal is generated by MHC-Antigenic Peptide Complex with T cell receptor (TCR), CD4 and CD8 co-receptors interaction [24]. The second, “costimulatory signal” is independent of antigen receptor, it comes from costimulatory membrane molecules, present in antigen-presenting cells (APCs), such as B7 molecules, whose ligands are found on the membrane of T lymphocytes. There is an “additional” level of regulation generated by the expression of inhibitory receptors that initiate intracellular transmission of negative signals. The balance between “negative” or inhibitory signals and “positive” or stimulatory signals, transmitted by antigen receptor, costimulatory and inhibitory molecules regulate the critical threshold level of T lymphocytes activation [24]. Costimulation involves a signal between cells that is reciprocal and sequential; lymphocytes that lack this signal when they encounter their antigen enter in energy state or die by apoptosis. Anergy consists in T or B lymphocytes clones impossibility to react against antigens, it is a mechanism of peripheral immune self-tolerance [24]. Costimulatory signals are emitted mainly by interactions between CD28 costimulatory receptor found on T lymphocytes surface and its ligands B7-1 (CD80) and B7-2 (CD86) expressed by APCs, in order to enhance T lymphocytes responses against antigens, including cell survival and proliferation signals, cytokines synthesis,

which allow cell-cell cooperation and naive lymphocytes differentiation into effector and memory lymphocytes. Costimulatory molecules expression increases in presence of microbial products (endotoxins) and cytokines such as Interferon-gamma (IFNγ).

B7 costimulatory molecules (B7-1 and B7-2) are transmembrane glycoproteins, which have extracellular domains similar to each other, but very different cytosolic domains. They are constitutively expressed in dendritic cells, and their expression is induced in activated macrophages and activated B cells. Mature dendritic cells express the highest concentrations of costimulatory molecules and are therefore potent naïve T cells stimulators [25]. B7 ligands are CD28 and CTLA-4, molecules expressed on T lymphocytes membrane. CD28 is constitutively expressed on T lymphocytes, in activated T cells induces IL-2 and the high-affinity IL-2 receptor expression. On the other hand, the inhibitory receptor CTLA-4 (CD152) is expressed on activated T cells surface and on regulatory cells Tregs, which play a fundamental role in the maintenance of peripheral self-tolerance, besides it has a higher affinity to bind B7 molecules (CD80 and CD86) compared to CD28. The B7-1 and B7-2 molecules expression patterns on cell surface of APCs are different, B7-2 is constitutively expressed and its expression increases rapidly after activation, whereas the B7-1 molecule is only expressed after activation. Based on these differences and various investigations, different authors conclude that B7-2 is the main ligand of CD28 (co-stimulator), and B7-1, the main ligand of CTLA-4 (inhibitor) [26]. In PMN neutrophils, B7 molecules have been observed to be stored in their cytoplasmic granules and under certain stimuli they are expressed on cell surface [27]. As mentioned above, costimulatory molecules B7-1 (CD80) and B7-2 (CD86) colocalization in NETs has been described [8, 9]. The importance of this finding is due to the fact that it has implications for the possibility of breaking immunological tolerance and would help explain diseases pathophysiology where these molecules are involved. It should be noted, in addition to two signaling pathways for cell activation described above, the existence of a third signalling pathway, given by cytokines generated as a product of infection and inflammation [25].

Regarding the interactions between T cells and neutrophils, IL17A, IL17F cytokines secreted by Th17 cells (which play a very important role in infections against extracellular bacteria and fungi) induce a rapid and massive infiltration of affected tissue by neutrophils. Reciprocally, neutrophils release chemokines that attract T cells to sites of inflammation and also cytokines that influence T cell differentiation and proliferation. Interestingly, a subset of neutrophils in human systemic inflammation inhibits T cell functions via MAC-1 [28]. B7 CD80/CD86 costimulatory molecules presence, colocalized in NETs [8], could provide a new function of neutrophils, giving to them possibility of APCs competences and modulation of T lymphocyte's different subpopulations functions. In addition, neutrophils isolated from synovial fluid of rheumatoid arthritis patients, express MHC class II molecules [29]. B7:CD80 costimulatory molecules cytoplasmic reservoirs have been described mainly within secretory vesicles and CD86 within secondary azurophilic granules and secretory vesicles [30]. In mouse models, neutrophil differentiation into a hybrid population exhibited a dual phenotype between neutrophils and dendritic [31]. Other authors have described the presentation of antigens via class II, by neutrophils [32]. In patients with Chagas disease-positive serology, contact interactions between neutrophils and lymphocytes have been observed [33].

CD4 is a co-receptor for helper T cells and primary receptor for HIV virus [34]. Various phenotypes of neutrophils have been [35] and it has been strikingly observed that peripheral blood PMNs neutrophil can unconventionally express CD4 molecule in a superficial or cytoplasmic form, in healthy patients and in HIV-positive patients [34]. Medical importance of these findings lies in possibility of influencing HIV biodistribution [34]. On the other hand, higher CD4 expression was observed in culture samples of healthy human PMN neutrophils stimulated with fMLP [36]. Regarding CD45RO molecule, a characteristic marker of memory and effector T cells, it is a tyrosine phosphatase that regulates lymphocyte activation [37], it is also expressed in PMN neutrophils of healthy people, but its natural ligand has not been found [38]. In addition, this molecule has been reported to be expressed in PMNs neutrophil from dialysis patients and PMNs neutrophil activated *in vitro* with fMLP [37]. CD45RO is found on neutrophil PMNs specific granules and it is possible that phosphatase activity of this molecule is involved in PMNs neutrophil adhesion during activation [37]. B7 costimulatory molecules of ETs could induce activation of CD4 T lymphocytes and influence he expression of CD45RO molecules in neutrophils.

Materials and Methods

Human blood samples: Heparinized human blood samples (n = 10) were collected with ethical consent according to procedures approved by ethical committee of National Hospital Clinicals. 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).

Autologous Total Leukocyte Cultures: Total leukocyte cultures were performed from human blood. Cells were grown in suspension, in sterile 24-well culture plates (some samples with sterile coverslips on the bottom) and others in culture tubes, in a gassed oven at 37 °C in TC199 medium (with Earle's salts and L-glutamine) (SIGMA, St. Louis, MO) added with serum from the same donor. The classic 0.5% Trypan Blue exclusion test for cell viability was used. All cell cultures were prepared under sterile conditions under a hood equipped with ultraviolet light and laminar flow. Samples were taken at 30 min. Paired autologous assays were performed with and without LPS stimulation (controls).

ETs generation: Cells cultured in medium with serum from same donor were stimulated: with LPS (Lipopolysaccharides of Escherichia coli, Sigma-Aldrich) 25 ng/ml; to form ETs at 37 °C in a gassed oven. Culture samples were taken at 30 minutes and isolation was performed. ETs were visualized with fluorescence microscopy using DAPI (4, 6'-diamidino-2-phenylindole) (Sigma, St Louis, MO) for DNA staining.

ETs isolation: After stimulation with 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. A 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 micro-centrifuged 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 [39].

Assay of B7 molecules in ETs influence on CD45 RO expression in neutrophils and in TCD4 cells: Paired autologous cultures of total leukocytes were subjected to cell-free ET stock according to ETs isolation assay. Samples of said cultures were taken at 24 hours and 72 hours to process with IF and perform CD45RO labelling.

Immunofluorescence (IF): Pelleted cultured cells were washed briefly in PBS (phosphate buffered saline), fixed in 4% paraformaldehyde 10 minutes and washed three times in PBS. They were incubated with "blocking serum" 5% albumin in PBS to prevent non-specific staining for 20 minutes. They were washed in PBS. ETs isolated samples were incubated with antibodies (Ab) Santa Cruz Biotechnology anti-CD80 (FITC; Santa Cruz Biotechnology), anti-CD86 (PE; Santa Cruz Biotechnology). Cell culture assay samples with and without interaction with ETs were incubated with anti-CD4 (PE; Santa Cruz Biotechnology) or with anti-CD45RO (FITC; Santa Cruz Biotechnology) at 4°C overnight. It was washed with PBS and DAPI (4,6'-diamidino-2- phenylindole) (Sigma, St Louis, MO) nuclear staining was performed in all cases. It was mounted with a 90% glycerol mounting medium in PBS. Observations were made using an Axioscop 20, MC80, trinocular, Carl Zeiss video microscope. Controls: paired samples. CD80 and CD86 IF in ETs isolated samples: recorded as positive or negative.

IF-labeled cells are positive for CD45RO and CD4 quantification in samples of human total leukocytes in autologous cultures in paired samples: positive cells percentage was calculated as a mean value in four fields (1000x) normalized by a total number of cells visualized with DAPI nuclear staining. Data were expressed as mean value ± SD. FIJI software was used [40].

Statistical analysis:_Student's t-test was used for paired samples. Infostat statistical program was used for its analysis [41].

Results

B7 costimulatory molecules colocalized in ETs influence on T CD4 lymphocytes activation and CD45RO molecules expression in human neutrophils in autologous culture was studied.

A) ETs generation in human leukocytes *in vitro* and isolation.

Autologous cultures of human whole blood leukocytes were performed from samples obtained as described in Material and Methods. These cell cultures were stimulated to generate ETs with LPS. ETs isolation was carried out and ETs generated were visualized with fluorescence microscopy as diffuse staining or fibrillar appearance using DAPI for DNA staining (Figure 1).

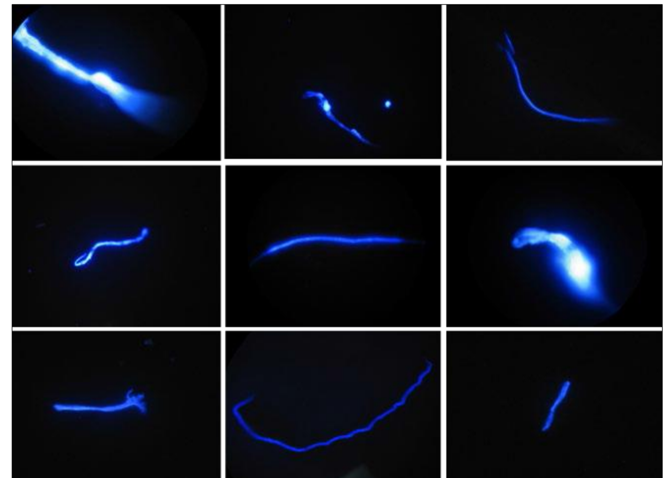

Fig 1: ETs generated by LPS stimulation. Representative image of IF, cell-free stock from autologous culture paired control samples of total leukocytes stimulated with 25 ng/ml LPS (Lipopolysaccharides from Escherichia coli, Sigma-Aldrich). 400 x. DNA staining with 4,6'-diamidino-2-phenylindole (DAPI) (blue).

B) Immunofluorescence labeling of B7 molecules in ETs

When B7 costimulatory molecules IF technique was performed in cell-free stock of ETs (Figure 2), ETs presence was observed with DAPI staining (Figure 2. A and D). B7-1 CD80 (Figure 2. C and F) and B7-2 CD86 (Figure 2. B and E) costimulatory molecules expression was observed.

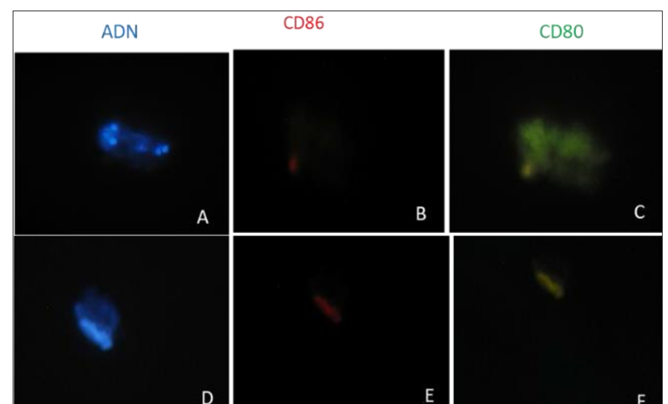

Fig 8: Expression of B7 costimulatory molecules (CD80 and CD86) in the cell-free stock of ETs.

Representative fluorescence microscopy images. 25 ng/ml LPS (Lipopolysaccharides from Escherichia coli, Sigma-Aldrich) 25 ng/ml.400x. A, B and C correspond to donor No. 1. D, E and F to donor No. 2. DNA staining with 4,6'-diamidino-2-phenylindole,
DAPI (blue), anti- CD86 (red) (PE; Santa Cruz Biotechnology), anti-CD80 (green) (FITC; Santa Cruz Biotechnology).

C) Immunofluorescence labeling of CD45RO and CD4 molecules in ET interaction with total leukocytes assay

Paired autologous cultures of total leukocytes were performed without ETs (controls) and with ETs addition. 24 hours of culture CD4 and CD45RO IF technique was performed on paired culture samples of total leukocytes at 24 hours of culture, without addition (controls) and with ETs addition generated by LPS (Figure 3).
Representative images of immunofluorescence microscopy. ETs generated with LPS (Lipopolysaccharides from Escherichia coli, Sigma-Aldrich) 25 ng/ml. A, B and C correspond to the control of donor No. 1. D, E and F correspond to the culture of the same donor with the addition of autologous ETs. G, H and I correspond to the control of donor N°2 and J, K and L correspond to the culture of donor N°2 and have added autologous ETs. DNA (blue) and expression of CD4 (red) and CD45RO (green) are observed. Anti-CD4 (PE; Santa Cruz Biotechnology) and anti-CD45RO (FITC; Santa Cruz Biotechnology) antibodies. DNA staining with 4,6'-diamidino-2-phenylindole (DAPI). The scale bar represents 10 µm.
In paired samples (Figure 3) at 24 hours of culture, cells with their DNA stained with DAPI were observed in control and in ETs addition samples (Figure 3. A, D, G and J). In control samples corresponding to donor No. 1, CD4 (Figure 3. B and H) and CD45RO (Figure 3. C and I) expression was not observed. In ETs addition samples, CD4 (Figure 3 E and F) and CD45RO (Figure 3. K and L) positive cells was observed after 24 hours of culture.
No significant differences were found according to Student's t-test in positive CD4 and CD45RO cells percentage between ETs addition samples and control samples, in all donors at 24 hours of culture. (Figure 4 and 5).

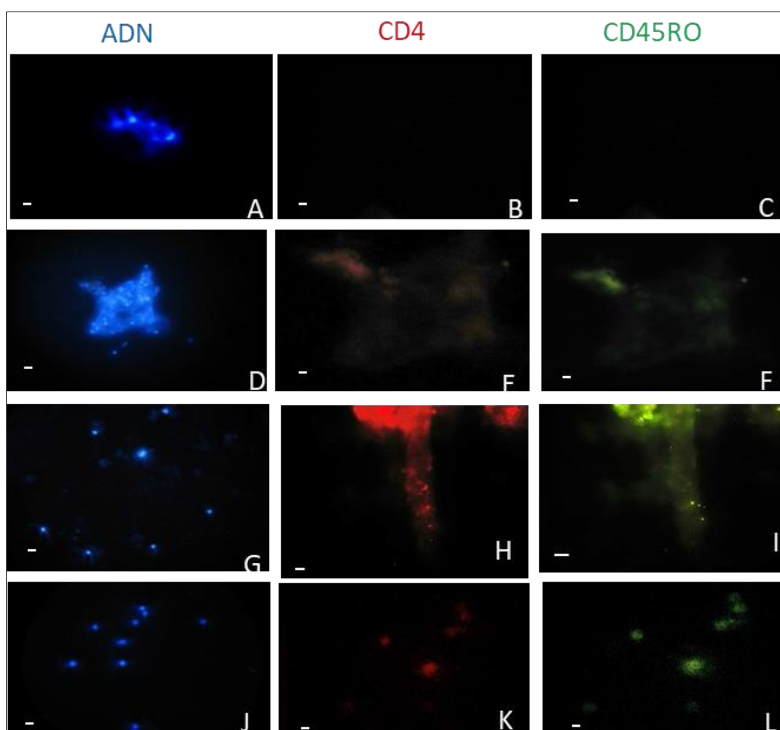

Fig 3: Expression of CD4 and CD45RO in paired autologous human total leukocyte culture samples without aggregate (controls) and with an aggregate of autologous ETs (Extracellular traps). 24 hours of culture.

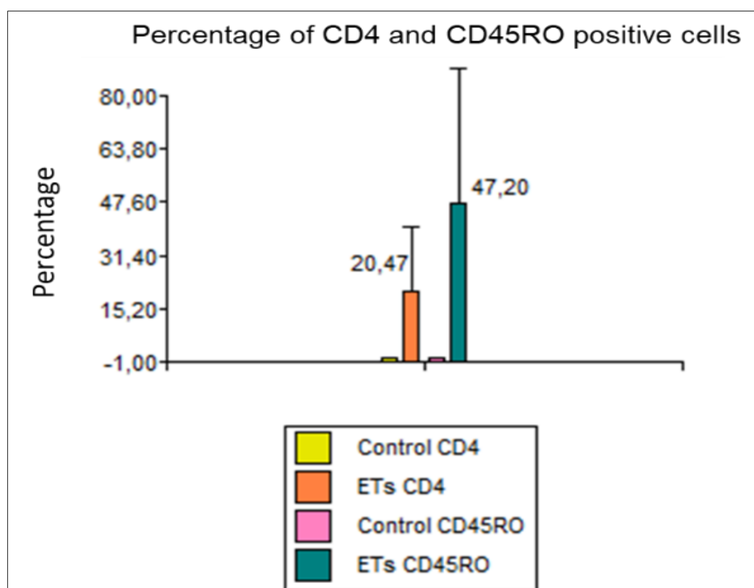

Fig 4: Percentage of CD4 and CD45RO positive cells in samples of human total leukocytes in autologous cultures.

24 hours of culture. Corresponds to donor No. 1. Control samples without added ETs. ETs: samples with aggregates of autologous ETs (extracellular traps). Data are presented as mean value ±SD ($p < 0.05$) Student's t test for paired samples. No significant differences were observed between the paired control samples and those with the addition of autologous ETs.

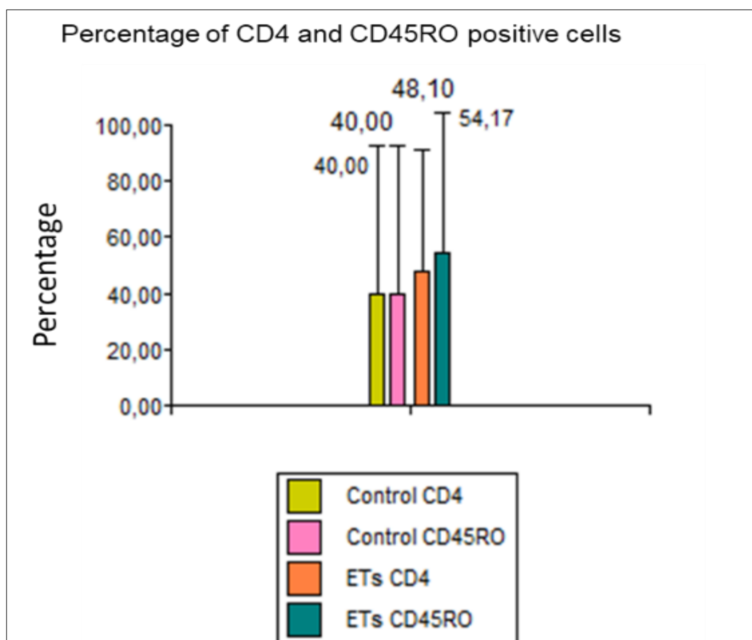

Fig 5: Percentage of CD4 and CD45RO positive cells in samples of human total leukocytes in autologous cultures.

24 hours of culture. Corresponds to donor No. 2. Control samples without added ETs. ETs: samples with aggregates of autologous ETs (Extracellular Traps). Data are presented as mean value ± SD ($p<0.05$) Student's t-test for paired samples. No significant differences were observed between the paired control samples and those with the addition of autologous ETs.

72 hours of culture

CD4 and CD45RO IF technique was performed on paired culture samples of total leukocytes at 72 hours of culture, without addition (controls) and ETs addition generated with LPS (Figure 6).

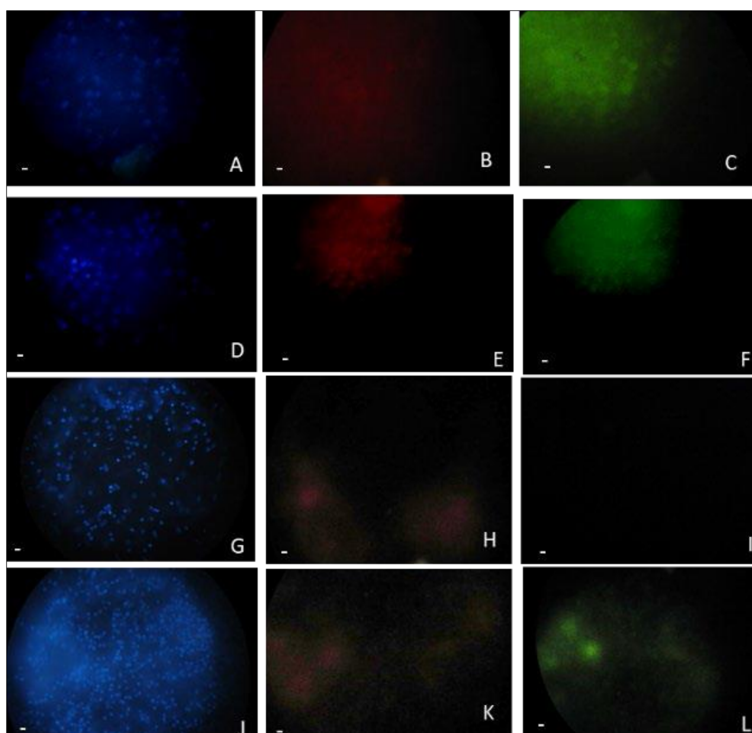

Fig 6: Expression of CD4 and CD45RO in paired autologous human total leukocyte culture samples without aggregate (controls) and with an aggregate of autologous ETs (extracellular traps).

72 hours of culture. Representative images of immunofluorescence microscopy. ETs generated with LPS. A, B and C correspond to donor control No. 2. D, E and F with added ETs from donor No. 2. G, H and I control donor No. 3. J, K and L with added ETs correspond to donor No. 3. DNA (blue) and expression of CD4 (red) and CD45RO (green). Anti-CD4 (PE; Santa Cruz Biotechnology) and anti-CD45RO (FITC; Santa Cruz Biotechnology) antibodies. DNA staining with 4, 6'-diamidino-2-phenylindole (DAPI). The scale bar represents 10 µm. After 72 hours of autologous culture, from paired samples, the presence of cells whose DNA was stained with DAPI was observed in control and in ETs addition samples (Figure 6. A, D, E, and J). In the first donor control samples, CD4+ (red) and CD45RO+ (green) cells were evidenced. In ETs addition samples, a significant increase was observed according to the t-test ($p < 0.05$), in CD4 and CD45RO expression on cells of the first donor (Figure 7).

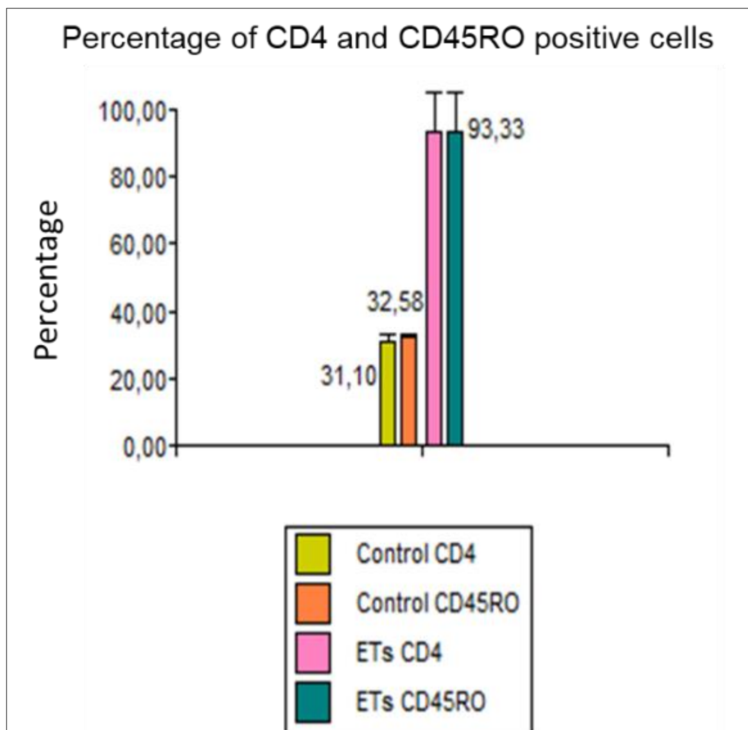

Fig 7: Percentage of CD4 and CD45RO positive cells in samples of human total leukocytes in autologous cultures.

72 hours of culture. Corresponds to donor No. 1. Control samples without added ETs. ETs: samples with aggregates of ETs. Data are presented as mean value ±SD ($p<0.05$) Student's t-test for paired samples. Significant differences are observed between the paired control samples and those with the addition of ETs.

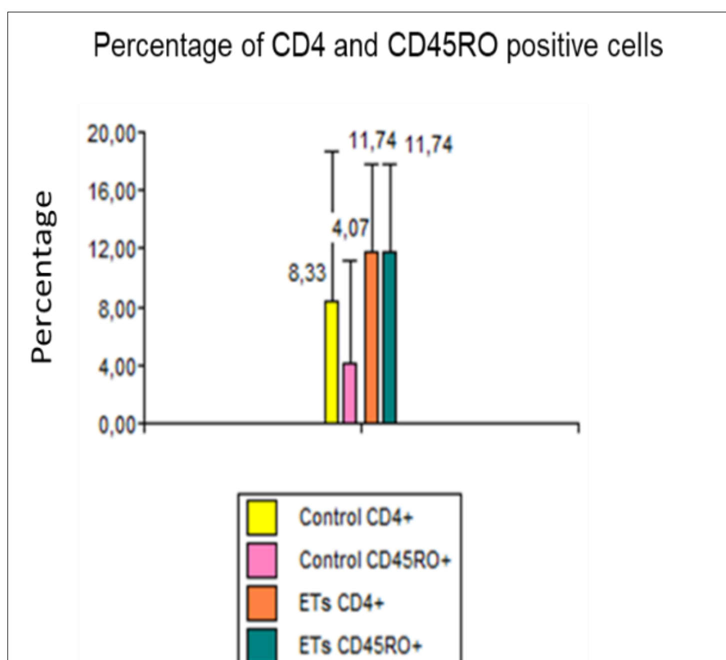

Fig 8: Percentage of CD4 and CD45RO positive cells in samples of human total leukocytes in autologous cultures.

72 hours of culture. Corresponds to donor No. 2. Control samples without added ETs. ETs: samples with aggregates of ETs. Data are presented as mean value ±SD ($p<0.05$) Student's t-test for paired samples. Significant differences are observed between the paired control samples and those with the addition of ETs in the expression of CD45RO.

In addition, control samples of the second, cells were positive for CD4 and negative for CD45RO (Figure 6. H and I) but when adding ETs in samples corresponding to the same donor, it was observed a significant increase according to t-test ($p< 0.05$), in CD45RO expression (Figure 6. L and Figure 8).

Discussion

The study of ETs generated by immune cells has currently received special attention. NETs generated to trap germs have also been involved in various diseases' pathogenic mechanisms, as mentioned above, and in particular in COVID-19, they have been involved in the immunothrombosis phenomenon due to interactions with platelets. Autopsies have confirmed pulmonary microthrombi containing NETs [42]. On the other hand, in the case of studies about NETs' influence in cancer, it has been described that they promote tumour growth and they have been directly implicated in the promotion of "exhausted" T cells in tumor microenvironment. These cells are characterized by overexpression of inhibitory receptors, decreased cytolytic activity and production of effector cytokines [43]. ETs can sometimes be considered sources of damage-associated molecular patterns (DAMPs). Said proinflammatory endogenous molecules are promoted by the released traps and are associated with various disorders in sepsis and cancer phenomena [44]. Studies are currently being oriented that take into account the source of DAMPs origin as a therapeutic strategy. Research on sepsis pathogenesis and pathophysiology has focused especially on NETs and their relationship with DAMPs [45]. Free DNA in extracellular space is considered a DAMP itself [46], in addition to DNA and histones released in NETs, MPO and NE also function as DAMP generators in an inflammatory tissular environment [44]. Through the pattern recognition receptors (RRPs) signalling by interaction with pathogen-associated molecular patterns (PAMPs) and DAMPs, an immune response is triggered.

As previously described in introduction of this work, ETs and especially NETs, when their excessive formation is not resolved, they are linked to numerous diseases.

We know T cell activity can be regulated, and having account about unconventional molecules expression on neutrophils, such as CD4 and CD45RO, besides costimulation of T lymphocytes possibility through B7 molecules (CD80 and CD86) released by ETs, is that objectives in this work were proposed. In this scientific research, we study B7 costimulatory molecules influence colocalized in ETs on CD4 T lymphocytes activation and CD45RO molecules expression in human neutrophils in autologous culture. As previously stated, CD45RO molecule is a tyrosine phosphatase that regulates lymphocyte activation and is considered a characteristic marker of effector and memory T cells [37]. CD45RO expression is not stable, loss of CD45RO expression induced by activation, with CD45RA maintenance during long-term cultures of T cells and NK cells, has been reported [47]. Although CD45RO is found in PMNs neutrophil-specific granules from healthy people, its natural ligand has not yet been found [38]. Chemotactic factors produce PMNs neutrophil activation *in vitro* mobilizing CD45RO to plasma membrane [48, 49]. It was early hypothesized CD45RO phosphatase activity might be involved in neutrophil PMN adhesion during activation [37].

Our hypothesis was that B7 costimulatory molecules released by ETs could induce CD4 T lymphocytes activation and also influence CD45RO molecule expression in neutrophils.

After LPS stimulation in total leukocytes autologous cultures for ETs generation, their isolation was carried out, producing the cell-free ETs stock [39] for interaction assays with autologous cultures in paired samples (Figure 1). B7 molecule labeling in autologous ETs was achieved in all experiments, result was recorded as positive (Figure 2). B7 costimulatory molecules (CD80 and CD86) expression in the cell-free stock of ETs was recorded in coincidence with works published by our laboratory [8]. In interaction assays of ETs with total leukocytes autologous culture at 24 hours, although variability between different donors is notable, no significant differences were observed in T-cell activation marker molecule (CD45RO) expression between paired control samples and ETs addition samples (Figures 3-5). Regarding the results of CD4 labeling, in one donor (donor Nº 1) it was observed as negative in controls (Figures 3 and 4). Although CD4 is a typical Th cell co-receptor, some PMN neutrophil phenotypes express as an unconventional molecule, on the other hand, it has been identified in very low expression by human monocytes [50]. In one study on its expression by monocytes, negative regulation of CD4 expression after isolation and culture has been observed [51]. We open a question regarding CD4 positive cell absence images in one of the donor culture samples (donor Nº 1) (Figures 3 and 4).

Differences observed in results obtained from one of the donor's paired samples (donor Nº1) respect to other donors' paired samples, calls attention related to possible interpersonal differences between donors. In this work, we considered as inclusion criteria, serological and molecular biology tests negativity that are carried out at IHH of UNC Blood Bank, like a "healthy" donor, suitable for blood transfusions. Diversity of factors that can influence immune responses must be taken into account when avoiding extrapolations of results obtained.

Analysis of results about ETs with total leukocytes in autologous culture interaction tests allows us to infer that after 72 hours of time, there were changes in molecules expression under study. Regarding CD4, a significant increase was observed according to t-test ($p< 0.05$), in CD4 expression in one of the donors (donor Nº1). On the other hand, after 72 hours of culture in all donors, significant differences could be observed in T cell activation marker molecule (CD45RO) expression (Figures 6 and 7). At this culture time, CD45RO-positive cells are considered to correspond to activated T lymphocytes.

The presence of colocalized CD80 and CD86 molecules in autologous ETs may influence various T cells activation. It is known more than one signal is necessary for naïve T cells activation since in addition to specific antigen recognition, costimulation and contextual cytokines play an important role [25]. As previously stated, antigenic recognition with costimulation absence causes energy or lack of immune response. However, presence of costimulatory molecules in

ETs could provide the second signal required for autoreactive lymphocytes activation, thus influencing possibility of peripheral tolerance breakdown. It should be noted different subpopulations in different functional states circulate in peripheral blood as well as naïve cells, and CD4+ memory, effector T cells, CD8+ memory T cells are positive for CD45RO. As described above, by alternative splicing the CD45RO isoform is expressed in membrane when cells are activated, making T cells more sensitive to stimulation with lower concentrations of MHC: Antigenic peptide complex, facilitating recognition. This occurs in memory and effector cells [25].

It should be noted in this research work it was decided not to use heterologous or homologous antigens in interaction tests between ETs and autologous leukocytes in culture. Results obtained after 72 hours of autologous culture with significant differences in CD45RO activation marker expression between controls and ETs addition can be explained due to the fact ETs present diverse components, like free DNA which acts as DAMP triggering an autologous immune response and, on the other hand, presence of co-localized B7 costimulatory molecules in traps could give a second signal required for autoreactive T lymphocytes activation present in autologous culture. This implies possibility of breaking self-tolerance.

Conclusion

At 72 hours of paired samples autologous culture, significant differences ($p<0.05$) were observed between controls and ETs addition samples in CD45RO expression. B7 molecule's presence in ETs could give a second signal required for autoreactive T lymphocytes activation present in total leukocytes autologous culture, implying possibility of breaking self-tolerance. NETs are implicated in various disease pathogenesis, including infectious diseases, autoimmune diseases and cancer, so contribution to new knowledge about their composition and influence on various cell types can contribute to their potential therapeutic targets.

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Conflict of Interest

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

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