Neutrophil Extracellular Traps Reprogram IL-4/GM-CSF-Induced Monocyte Differentiation to Anti-inflammatory Macrophages

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Monocyte-derived dendritic cells (mo-DCs) are essential for the development of a Th1 protective immune response against *Leishmania* parasites. It is well known that IL-4 and GM-CSF drive differentiation of human monocytes to dendritic cells (DCs). Here, we investigate if neutrophil extracellular traps (NETs) disrupt this process. NETs-enriched supernatants, generated after human neutrophil activation by *Leishmania* promastigotes, were added to monocytes and differentiation monitored by expression of molecules associated with macrophage and DCs phenotypes, cytokine production, and parasite killing. We found that NETs addition to IL-4/GM-CSF-treated monocytes prevented then to fully differentiate into DCs. No effect was observed if NETs were treated with DNase or by filtering the traps. Moreover, NETs closely interact with monocytes and downregulate the expression of the IL-4 receptor, which in turn disrupts fully differentiation of monocytes into DCs. Neutrophil elastase inhibition rescues the monocytes to DCs differentiation. Monocytes cultured with IL-4/GM-CSF and NETs differentiated into macrophages, as observed by the increased expression of CD68, CD32, and CD163, and decreased expression of CD80. Moreover, NET addition to IL-4/GM-CSF-treated monocytes rendered these cells less efficient to kill *Leishmania* parasites. Altogether, our results show that NETs interfere with IL-4/GM-CSF driven differentiation, reprogramming the generation of mo-DCs to an anti-inflammatory macrophage.

**Keywords:** neutrophil extracellular traps, monocytes, IL-4 receptor, elastase, dendritic cells, *Leishmania*

INTRODUCTION

Neutrophils are endowed with several antimicrobial proteins and upon activation can kill microorganisms by the release of the neutrophil extracellular traps (NETs) (1). NETs are web-structures composed by DNA and proteins from different neutrophil compartments, such as histones and elastase, functioning in the contention and killing of microorganisms (1, 2). Although the hallmark of NETs is the ability to confine and kill pathogens, many studies have reported that NETs can orchestrate the immune response during autoimmune diseases (3–9). NETs-activated dendritic cells (DCs) induce anti-neutrophil cytoplasmic antibody production and autoimmunity in naïve mice, a feature
prevented by NET digestion with DNase (3). NETs were found to be the major inducer of type I interferon (IFN) production by plasmacytoid DCs in patients with systemic lupus erythematosus, meaning that NETs can modulate DCs function (5–7). To date NETs were described to activate both pro-inflammatory and anti-inflammatory responses in other immune cell types. NETs formed upon activation of neutrophils with *Mycobacterium tuberculosis*, interact with and activate macrophages, inducing IL-6, tumor necrosis factor-α (TNF-α), IL-1β, and interleukin-10 (IL-10) production (10), and NETs from cholesterol crystal-stimulated neutrophils prime monocytes to produce IL-6 and IL-1β in response to these cholesterol crystals (11). Contrariwise, NETs inhibit the activation of monocyte-derived dendritic cells (mo-DCs) by lipopolysaccharide (LPS) and promote polarization to a Th2 immune response (12).

During *Leishmania* infection, neutrophils are one of the first lines of defense and the first cells to be parasitized by *Leishmania* parasites (13, 14). Leishmaniasis is a neglected tropical disease transmitted by bites of phlebotomine sand flies, which inoculates parasites along with saliva, rousing a massive and rapid influx of neutrophils, followed by monocytes to the inoculation site, where these cells are likely to interact (13, 14). Our group has demonstrated that *Leishmania* activate NETs formation, and parasites are ensnared and killed by NETs-associated histones (15). However, a portion of the *Leishmania* population, despite its ability to induce NETs release, evade NETs-mediated killing (16–18). Moreover, saliva of the *Leishmania* vector, *Lutzomyia longipalpis*, contains a potent nuclease that digests NETs structure allowing the parasites to escape NET-mediated killing (18).

Recently, analysis of 35 biopsies of patients with cutaneous leishmaniasis showed that NETs were presented in 77.1% of the biopsies. Furthermore, amastigotes were observed ensnared by the traps (19).

Depending on the microenvironment, monocytes can differentiate into DCs or macrophages. Dermal infection by *Leishmania major* leads to the differentiation of mo-DCs in the inflammatory site, which are infected by the parasite (20). Interestingly, dermal-differentiated DCs produce large amounts of IL-12 and stimulate a specific T cell response. Moreover, DCs differentiated from monocytes at the site of *Leishmania* infection produce large amounts of nitric oxide (NO), an important mediator for parasite killing (21). Additionally, in *vitro* infection with *Leishmania amazonensis* impairs human monocytes to differentiate into DCs, weakening the induction of a proper Th1 cell response (22).

Pondering that neutrophils and monocytes could interact at the site of *Leishmania* infection, we raised the question whether NETs could impact monocytes differentiation into DCs, affecting the response to parasites. Our results clearly show that NETs impair fully differentiation of monocytes into DCs and downregulate the expression of IL-4 receptor on monocytes due to elastase activity. NETs intimately interact with monocytes and IL-4/GM-CSF-treated monocytes exposed to NETs are less efficient in *Leishmania* killing. Moreover, IL-4/GM-CSF-treated monocytes cultured in the presence of NETs exhibited increased expression of molecules and cytokines associated with an anti-inflammatory macrophage phenotype. Our results suggest that NETs interfere with monocyte differentiation, reprogramming the generation of mo-DCs to an anti-inflammatory macrophage phenotype.

**MATERIALS AND METHODS**

**Neutrophil and Monocyte Purification**

Human neutrophils and monocytes were isolated by density gradient centrifugation (Histopaque; Sigma-Aldrich) from buffy coats of healthy blood donors. PBMCs were collected and washed three times with PBS, resuspended in RPMI medium 1640 (Sigma-Aldrich) supplemented with Nutridoma-CS (1×; Roche Applied Science) and incubated for adherence (see below). In some experiments, monocytes were purified with the Monocyte Isolation Kit II (Miltenyi Biotec) according to the manufacturer’s instructions.

The layer containing neutrophils was subjected to hypotonic lysis of erythrocytes. Purified neutrophils were resuspended in RPMI medium 1640 and kept on ice until use. Human PBMCs from healthy subjects were obtained under written informed consent and all procedures were approved by the Institutional Review Board for Human Subjects (Research Ethics Committee) from Hospital Clementino Fraga Filho, Universidade Federal do Rio de Janeiro (protocol number 055-15) and from the NIH Clinical Center IRB-approved protocol from the NIH Clinical Center Department of Transfusion Medicine (protocol number 99-CC-0168).

**Monocyte-DC Differentiation Assay**

PBMCs (5 × 10⁶; 1 mL) were incubated for 2 h at 37°C in 5% CO₂ to allow monocytes to adhere in 24-well plates. Non-adherent cells were washed out and the adhered monocytes were used throughout the experiments. NETs-enriched supernatants treated or not with DNase, or filtered were added and cultures maintained at 37°C in 5% CO₂. After 18 h, GM-CSF and IL-4 (50 ng/mL each; Peprotech) were added and the cultures maintained for 5 days at 37°C in 5% CO₂. Cells were then harvested, stained for CD1a (PE; BD), CD14 (FITC; BD), CD68 (FITC; BD), CD32 (PE-Cy7; BD), CD163 (APC; BD), and CD80 (APC-Cy; BD), and analyzed on a MACSQuant flow cytometer (Miltenyi Biotec). Cells were acquired based on forward and side scatter and data analyzed with FlowJo Software 10.0.8. All experiments with monocytes were done in medium supplemented with Nutridoma-CS (1×; Roche Applied Science). In some experiments, monocytes were pretreated with cytochalasin D (CytD) (10 µg/mL; Sigma) or the diluent DMSO for 30 min before the addition of NETs.

**Parasite Culture**

*Leishmania amazonensis* (WHOM/BR/75/Josefa) promastigotes were grown at 26°C in Schneider’s Insect Medium (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal calf serum (FCS; Cryopion, São Paulo, Brazil) and 40 µg/mL of gentamicin (Schering-Plough, Rio de Janeiro, Brazil). At days 5–6 of culture, stationary-phase promastigotes were obtained and used throughout the experiments. In parallel, *L. amazonensis* promastigotes were fixed with paraformaldehyde (4% in PBS) for 30 min at room temperature. Parasites were then extensively washed with PBS and resuspended in RPMI for further NET induction.
Production of NETs-Enriched Supernatants

Neutrophils (8 × 10⁶) were incubated with live or paraformaldehyde-fixed promastigotes of *L. amazonensis* in a 1:0.1 neutrophil:parasite ratio or LPS (Escherichia coli O55:B5) 100 ng/mL at 37°C in 5% CO₂. After 3 h incubation, supernatant was collected and aliquots were kept at −80°C until use. The quantification of NETs were performed with the Picogreen dsDNA kit (Invitrogen, Life Technologies), as already described (15). The NETs-enriched supernatants were treated with DNase (10 U/mL; Fermentas Life Science) or with the elastase inhibitor MeOSuc-aAPV-cmk (10 µg/mL, Calbiochem) for 30 min and then added to monocytes. In some cases, NETs were filtered through a 0.22 µm pore filter to remove the NET scaffold.

The quantification of the elastase in the supernatants was performed as described (18). Briefly, 50 µL of NETs-enriched supernatants were incubated with the fluorogenic substrate N'-methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin (0.25 mM; Sigma-Aldrich). After 1 h at 37°C, the fluorescence was measured in a SpectraMax Paradigm reader (Molecular Devices) at 365–450nm. A standard curve with recombinant elastase was used to determine the concentration of elastase in the NETs-enriched supernatants.

Cell Viability Assay

Adhered monocytes were treated or not with NETs-enriched supernatants as described above for 18 h, and lactate dehydrogenase (LDH) in the culture supernatant was measured according to the manufacturer’s directions (Promega). Briefly, 50 µL of culture supernatant was incubated with 50 µL of substrate mix in a 96-well plate at room temperature protected from light. After 30 min, 50 µL of stop solution was added, and the plate was read at 490 nm on a SpectraMax Paradigm reader.

Immunofluorescence

PBMCs (1 × 10⁶; 1 mL) were incubated for 2 h at 37°C in 5% CO₂ to allow monocytes to adhere to coverslips. Non-adherent cells were washed out and adhered monocytes were incubated with NETs-enriched supernatants (2 µg of DNA) for 4 h and fixed with 4% paraformaldehyde. Slides were stained with DAPI (10 µg/mL; Sigma), anti-elastase (1:800 v/v; Calbiochem), or anti-histone/DNA complex (1:150 v/v; Abcam), followed by anti-rabbit-FITC (1:150 v/v; Vector Labs) or anti-rabbit-Texas Red (1:150 v/v; Invitrogen), respectively. Epifluorescence images were taken in a Zeiss Axioplan using 40x objectives.

Phagocytosis and Leishmania Killing Assay

For the phagocytosis assay, promastigotes of *L. amazonensis* were labeled with the CellTrace™ CFSE Cell Proliferation Kit as recommended by the manufacturer (Invitrogen, Life Technologies). Briefly, promastigotes (1 × 10⁶ cells/mL) were incubated with 2.5 µM of CFSE in pre-warmed (37°C) PBS. After 10 min at 37°C, five volumes of ice-cold RPMI were added in order to quench the staining and cells were incubated for 5 min on ice. Parasites were then washed three times and resuspended in RPMI. Monocytes were treated as described in the Section “Monocyte-DC Differentiation Assay.” After 18 h of NET treatment, monocytes were incubated with CFSE-labeled *L. amazonensis* promastigotes at a 1 monocyte:3 parasites ratio. After 4 h of incubation at 37°C in 5% CO₂, monocytes were harvested, washed and cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Monocytes were acquired based on forward and side scatter and data were analyzed with Summit Software 4.3. CFSE-positive monocytes were considered as infected cells.

For the parasite killing assay, adhered monocytes were incubated with NETs, treated or not with nuclease or elastase inhibitor for 18 h at 37°C in 5% CO₂. Promastigotes were added to the culture in a 1 monocyte:3 parasites ratio and the coculture was maintained overnight at 35°C/5%CO₂. Non-phagocytosed parasites were washed out. After 48 h of infection, monocytes were lysed with 0.01% sodium dodecyl sulfate (SDS) for 5 min at room temperature. Lysis was stopped by addition of Schneider medium supplemented with 10% FCS (Schneider complete medium), and parasites allowed to differentiate and grow for 48 h. Viable and motile promastigotes were counted in a Neubauer chamber.

Cytokines Quantification

Monocytes were treated with NETs-enriched supernatants as described in the Section “Monocyte-DC Differentiation Assay.” After 5 days, cells were activated with LPS (100 ng/mL; Sigma) and cell culture supernatant was collected after 72 h and kept at −80°C until use. TNF-α, interleukin-12p40 (IL-12p40), transforming growth factor-β (TGF-β), and IL-10 concentrations were quantified by ELISA (Duoset Kits; R&D Systems) according to manufacturer’s instructions.

Expression of IL-4 and GM-CSF Receptors

Adhered monocytes were incubated or not with NETs-enriched supernatants (2 µg of NET-DNA), treated or not with nuclease or the elastase inhibitor methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (MeO Suc-AAPV-cmk; Calbiochem) and cultures maintained at 37°C in 5% CO₂. After 18 h, cells were harvested, incubated with FcR blocking reagent (Miltenyi), and stained with anti-IL-4 receptor α chain (PE; R&D) and anti-GM-CSF receptor (APC; R&D) antibodies. Cells were acquired based on forward and side scatter and analyzed using a FACSCalibur flow cytometer (BD) and data were analyzed with Summit Software 4.3.

For real-time PCR quantification of the expression of the IL-4 receptor, monocytes (1 × 10⁶ cells), purified with the Monocyte Isolation Kit II (Miltenyi Biotec), were treated or not with digested or non-digested NETs (dNETs)-enriched supernatants. After 4 h of treatment, RNA was extracted from the cell pellets, using the RNeasy mini Kit (Qiagen, Valencia, CA, USA) and treated with DNase I. cDNA synthesis was performed using the qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD, USA). Genomic DNA contamination was measured by PCR of total RNA. Relative quantification of the IL-4 receptor expression was performed in a LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA) using the Universal ProbeLibrary system...
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**Western Blot Assay**

IL-4 receptor cleavage was analyzed by Western blot. Recombinant soluble Human sIL-4 Receptor α (200 ng; Peprotech) was incubated with NETs (160 ng of NET-DNA), pretreated or not with 10 µg/mL of elastase inhibitor, or with recombinant elastase in different concentrations for 30 min at 37°C. Ten microliters were submitted to 10% SDS-PAGE. Western blot was carried using a Human IL-4R α biotinylated antibody (0.1 µg/mL; R&D) followed by avidin–alkaline phosphatase (1:150,000 v/v; Sigma-Aldrich) incubation. blot was revealed with Western Blue stabili-

**Data Analysis**

Results are expressed as mean ± SEM and P < 0.05 was considered significant. For multiple comparisons, One-way ANOVA analysis followed by Fisher’s least significant difference was performed. Paired t-test analysis was performed for some experiments as indicated in the figure legend. Data analysis was performed by GraphPad Prism 5.03 software.

**RESULTS**

**NETs Impair Fully Maturation of Monocytes into DCs**

Neutrophil extracellular traps-enriched supernatants (here referred as NETs) were added to adhered monocyte culture 18 h before treatment with IL-4/GM-CSF, and differentiation of monocytes into DCs was analyzed by the surface membrane expression of CD1a and CD14 by flow cytometry. Human monocytes are CD14+CD1a- (Figures 1A–E) and, during the IL-4/GM-CSF-driven in vitro differentiation process, their expression of CD14 decreased, whereas the expression of CD1a increased, as expected. Treatment with NETs before the addition of IL-4/GM-CSF, lead to a reduction in the percentage of CD1a+ cells by 42%, with a 79% reduction of the MFI (Figures 1B,C), relative to control (cells treated only with IL-4/GM-CSF). Expression of CD14 was partially maintained by NET treatment with 20% of CD14+ cells, a 7.7 times higher expression compared to control (2.6% of CD14+ cells) (Figure 1D). In addition, CD14 increased 2.7 times in monocytes treated with NETs compared to control (Figure 1E). Moreover, the effect of NETs on monocyte differentiation is dose-dependent (Figure 1F), and treatment with DNase-dNETs or filtered-NETs had no effect on monocyte differentiation into DCs (Figures 1A–E). As portrayed, monocytes intimately interacted with NETs, which were visualized surrounding groups of monocytes. It is worthy to point out that NETs integrity was still maintained during the recovery of NETs-enriched supernatants. Furthermore, treatment with NETs-enriched supernatants was not toxic to monocytes, as assessed by extracellular LDH measurement (data not shown).

Because it has been reported that NETs interact and are endocytosed by human macrophages (23), we asked whether blockage of NETs uptake by monocytes would inhibit NETs effect on monocyte differentiation. Our results show that NETs were still able to impair mo-DCs generation even after endocytosis inhibition by CytD treatment (Figure 1H).

**NETs Downregulate IL-4 Receptor Expression**

It was previously reported that neutrophil elastase cleaves G-CSF receptor (24). Since elastase is one of the NETs components, we asked whether NETs could also downregulate the expression of GM-CSF or IL-4 receptors, thus impairing the differentiation of monocytes into DCs. Interestingly, we found that the expression of IL-4 receptor (α chain) was reduced by 38% in NET-treated monocytes in comparison with control (Figures 2A–C). Pretreatment of NETs with elastase inhibitor rescued the expression of IL-4Rα (Figures 2B,C), suggesting a role for this enzyme in NETs-induced reduction of IL-4Rα expression. DNase-dNETs did not change IL-4Rα expression (Figures 2B,C), and we did not observe any differences in the expression of GM-CSF receptors in monocytes treated with NETs (Figure 2A). Next, by real-time PCR analysis of IL-4Rα mRNA expression, we tested whether NETs-induced downregulation of IL-4Rα expression could also occur at a transcriptional level. We found that monocytes treated with NETs express 50% less IL-4R mRNA than control (Figure 2D), suggesting that the IL-4R mRNA synthesis or transcriptional control is altered in NETs-exposed monocytes. IL-4R mRNA expression was not affected by DNase-dNETs (Figure 2D). Interestingly, monocytes treated with commercial recombinant elastase did not show any difference in IL-4R mRNA expression, relative to non-treated cells, showing that the preserved structure of NETs might be crucial for this activity (Figure 2E). To evaluate whether NETs could cleave the IL-4R, we incubated commercial IL-4Rα with different concentrations of elastase, NETs, or elastase inhibitor-treated NETs for 30 min. Our results show that elastase and NETs cleave IL-4R and that pretreatment of NETs with 10 µg/mL

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NETs Block Monocytes Differentiation to DCs

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of elastase inhibitor prevented degradation of the receptor (Figures 2F,G).

**NETs-Treated Monocytes Differentiate into Macrophages**

Our results showing that monocytes treated with NETs before the addition of IL-4/GM-CSF were unable to fully differentiate into DCs led us to wonder whether these monocytes were being skewed into macrophages. Thus, we analyzed the expression of common surface and intracellular molecules of macrophages in the cells that emerged from the monocytes treated with NETs/IL-4/GM-CSF. We observed that NET-treated monocytes had an increased percentage of CD68+, CD32+, and CD163+ cells in comparison to monocytes cultured only with IL-4/GM-CSF (Figures 3A–C). However, NETs treatment diminished the percentage of CD80+ cells, suggesting that NETs can up- or downregulate monocyte membrane molecules. NETs disrupted by DNase treatment did not change the expression of CD32, CD80, and CD163 relative to IL-4/GM-CSF-treated cells (Figures 3B–D).
We also analyzed the secretion of cytokines by NET-treated cells, stimulated with LPS. Monocytes differentiated in the presence of NETs produced 78 and 70% less amounts of TNF-α and IL-12p40, respectively, than cells cultured with IL-4/GM-CSF only (Figures 4A,B). Although elastase and dNETs increased 2.3 times the amounts of TNF-α in relation to NETs/IL-4/GM-CSF-treated cells, they decreased around 49% TNF-α production in relation to control cells. Similarly, both elastase and dNETs treatments increased around two times IL-12p40 secretion in relation to NETs/IL-4/GM-CSF-treated cells, and decreased 25 and 37%, respectively, the IL-12p40 secretion in relation to control cells (Figures 4A,B). Inversely, NETs-treated monocytes produced significantly higher amounts (twice as much for each cytokine) of TGF-β and IL-10 (Figures 4C,D). Monocytes treatment with dNET or elastase did not change IL-10 production compared to control cells (Figure 4C). Elastase treatment increased 2.2 times TGF-β amounts in relation to control cells and no difference was observed comparing elastase and dNET in relation to NETs/IL-4/GM-CSF-treated cells (Figure 4D). Hence, we can conclude that NETs treatment reprograms the IL-4/GM-CSF-induced monocyte differentiation to monocytes with an anti-inflammatory profile.

**NETs-Treated Monocytes Are Less Efficient in Parasite Killing**

To study a functional aspect of the generated cells, we evaluated the antimicrobial capacity of both kinds of differentiated monocytes, by measuring their ability to phagocytose *Leishmania* promastigotes. NETs did not interfere with the ability of IL-4/ GM-CSF-treated monocytes to bind parasites (Figure 5A). However, NETs-treated monocytes partially lost their capacity to kill parasites, compared to IL-4/GM-CSF-treated monocytes (Figures 5B,C). Importantly, this result was further confirmed in another set of experiments, in which NET treatment significantly decreased the ability to kill parasites of cells from seven different donors tested (Figure 5B, insert). The ability to kill parasites was preserved in dNETs- and elastase inhibitor-treated monocytes (Figures 5B,C). During the interaction with NETs-treated monocytes, parasite survival was equal to 70% higher, relative
Figure 3 Neutrophil extracellular traps (NETs)-treated monocytes differentiate into macrophages. Adhered monocytes were incubated or not with NETs-enriched supernatants or digested NETs (dNETs) for 18 h. IL-4 and GM-CSF were added to the culture (where indicated) and the cells were maintained at 37°C/5%CO2. (A–D) After 5 days, cells were harvested and stained for (A) CD68, (B) CD32, (C) CD163, and (D) CD80. Results of at least five experiments are shown as % of cells expressing the cell marker and as mean ± SEM. (A) *P < 0.05. (B) ****, ***, **P < 0.001; *P < 0.05. (C) ***, **P < 0.01; *P < 0.05. (D) **P < 0.05.

Figure 3 [Nettext]

** DISCUSSION **

Neutrophil extracellular traps have been extensively studied in the last decade. Since the first report, NETs have been suggested to participate in a great number of infectious and non-infectious diseases. It has been reported by our group and others that *Leishmania* parasites induce the release of NETs (15, 16, 25). We also demonstrated the presence of NETs structure in biopsies of patients with cutaneous leishmaniasis (15, 19). However, little is known about the impact of NETs on other immune cells, which prompted us to investigate whether NETs could affect human monocytes. Herein, we demonstrate that NETs downregulate the expression of the IL-4 receptor in monocytes, impairing the differentiation of these cells into immature DCs, and affecting killing of *Leishmania* parasites.

Impairing the generation of DCs derived from monocytes can prevent the generation of a protective immune response. DCs are the primary antigen-presenting cells and thereby connect the innate to the adaptive immune system. During infections, DCs...
are activated and enter into a process of maturation that involves changes in the ability to capture antigens, increased expression of costimulatory molecules, and migration to secondary lymphoid organs, where DCs activate T cells, controlling the quality of the Th1/Th2 immune response (26, 27). DCs play an important role in generating a protective Th1 response during *Leishmania* infection through the production of IL-12 (28), which is important for the infection control and the development of resistance. Th1 cells produce IFN-γ, which induces the expression of inducible nitric oxide synthase (iNOS) by phagocytic cells, leading to parasite killing (20). After migration through the endothelium, monocytes can differentiate into macrophages or DCs at the infection site (20). The mo-DCs are essential for the formation of a protective Th1 response during *Leishmania* infection. It has been shown that mo-DCs are formed at the site of *Leishmania* infection and constitute the main iNOS-producing cells, which is important for the elimination of parasites (20, 21). Nevertheless, during infection by *Leishmania mexicana*, the recruitment of monocytes to the site of infection is reduced and mo-DCs that are harvested from lesions produce low amounts of NO (29). Furthermore, it was demonstrated that the *in vitro* infection of human monocytes with promastigotes of *L. amazonensis* prevents the formation of mo-DCs, affecting the generation of a protective immune response (22). Our data demonstrate that NETs impair the process of monocytes differentiation into DCs and NETs digestion with DNase did not affect this differentiation process. The excessive NET formation at the site of *Leishmania* infection or a defect in the clearance of NET-structures could affect the generation of a protective Th1 response, which is important for the infection control. In fact, it has been demonstrated that during *L. mexicana* infection in C57BL6 mice, early neutrophil recruitment is associated with a decrease of monocyte and mo-DCs
recruitment (25). The authors also showed the presence of NETs in L. mexicana mouse ear infection (25). Future studies need to be conducted to evaluate the impact of NETs in generating mo-DCs in the in vivo model of Leishmania infection and its implication in the development of a Th1 immune response.

A likely explanation for the impairment in monocyte differentiation process is the modulation of the expression of receptors for GM-CSF and IL-4. It has been demonstrated that treatment of human neutrophils with recombinant neutrophil elastase decreases the expression of G-CSF receptor in a time-dependent fashion, as this enzyme can cleave G-CSFR (24). Digestion of the receptor was observed by detection of receptor’s fragments in culture of elastase-treated neutrophils (24). Since elastase is one of the major components of NETs, we investigated its effect in the expression of IL-4 and GM-CSF receptors. Interestingly, a decrease in the IL-4Rα expression was detected on NETs-treated monocytes, together with a decrease in the IL-4R mRNA, indicating that NETs induce not only digestion of the expressed receptor, as well as, exert a transcriptional regulation of this receptor. We have not detected any changes in the expression of GM-CSFR. Digestion of NETs fully abolished the elastase effect on the expression of the IL-4Rα on monocytes, indicating that NET structure must be intact to be effective. Moreover, this data indicates that degranulated neutrophil elastase would not have the same effect as NETs-associated elastase, since the structural integrity of NETs is required to regulate IL-4R expression. We have not established here the mechanism by which NETs downregulate IL-4R expression. However, it was demonstrated that type I IFN and IFN-γ diminish IL-4R expression on mononuclear cells and B cells purified from human peripheral blood (30). It was also demonstrated that both types of IFN post-transcriptionally downregulate IL-4R expression by affecting the mRNA stability (30). Besides, IFN-γ reduces the expression of both γc and α IL-4R chains on human monocytes cell surface (31). NETs were previously shown to be the major inducer of type I IFN production by plasmacytoid DCs during autoimmune disease (5), a possible explanation for NETs’ downregulation of the IL-4R would be through the production of IFN by NETs-treated monocytes.

The lower expression of the α chain of the IL-4R may have implications in the course of the disease. The protective immune response during Leishmania infection requires the activation of Th1 cells and production IFN-γ, which activate the macrophages’ microbicidal mechanisms. By contrast, the Th2 response with high production of IL-4 and IL-13 is associated with susceptibility to infection. BALB/c mice are susceptible to infection by L. major due to the development of a Th2 response to the parasite. BALB/c mice deficient in IL-4 or with the expression of IL-4Rα deleted specifically in CD4+ T cells become resistant to Leishmania infection (32, 33). In addition, treatment of L. major-infected mice with soluble IL-4R results in a decrease in parasite load in spleen and lymph nodes and promoted resistance to reinfection by L. major (34).

Despite the deleterious effect of IL-4 during Leishmania infection, contrasting data have been reported in the literature regarding the role of IL-4 during infection. Unexpectedly it has been demonstrated that administration of recombinant IL-4 8 h after infection with L. major promotes IL-12 production by DCs in vivo (35). However, when IL-4 was administered at later time points, C57BL/6 resistant mice become susceptible to L. major infection (35). These data demonstrate that IL-4 has different roles during the course of leishmaniasis. This interleukin seems to have a protective effect in the early post-infection events. Furthermore, deletion of the α chain of the IL-4 receptor on DCs turn BALB/c hypersusceptible to infection with L. major (36), indicating a protective role of IL-4...
Fig. 6 | Summary scheme for neutrophil extracellular traps’ (NETs’) blockage of dendritic cells (DCs) differentiation from monocytes. (A) In the presence of IL-4 and GM-CSF, monocytes are stimulated to differentiate into DCs. These cells express CD1a molecules and, when stimulated by lipopolysaccharide (LPS), produce high amounts of pro-inflammatory cytokines [IL-12 and tumor necrosis factor-α (TNF-α)]. (B) We show in this study that NETs, produced by *Leishmania amazonensis*-activated human neutrophils, downregulate the expression of the IL-4 receptor α chain skewing monocytes to differentiate into anti-inflammatory macrophages. Macrophages differentiated in the presence of NETs express CD68, CD163, and CD32 and, when stimulated by LPS, produce high amounts of interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), favoring *Leishmania* survival. 

During *Leishmania* infection, all these studies show that the role of IL-4 in *Leishmania* remains contradictory. In our study, we have not evaluated the production of IL-4 by monocytes treated with NETs. However, these cells expressed lower amounts of IL-4Rα on its surface. The absence of IL-4 signaling pathway in monocytes specifically has not yet been evaluated during *Leishmania* infection. Our data point to a lower parasite killing capacity of monocytes cultured in the presence of NETs. Further studies need to be conducted to correlate the expression of the IL-4R on monocytes treated with NETs with the monocyte microbicidal capacity.

Monocytes that were treated with NETs differentiated more in macrophages than in DCs, with expression of CD68, a macrophage marker, and classic macrophage morphology (a network of elongated cells with strongly adherence to plastic; data not shown). The analysis of surface markers of differentiated cells in the presence of NETs revealed that NETs-treated monocytes differentiated into macrophages with a profile of CD32⁺CD68⁺CD163⁺CD1a⁻ cells. Moreover, these cells have reduced expression of CD80 and increased expression of CD163 when treated with NETs, thereby characterizing an M2 macrophage, as established in the literature (37, 38). Studies in the literature suggest that NETs activate a pro-inflammatory response in other immune cells. These structures are capable of activating the production of type I IFN in plasmacytoid DCs and activate the production of pro-inflammatory cytokines (such as IL-1β, TNF-α, and IL-6) in macrophages (5–7, 10). However, different from what it was shown in the literature, in our experiments NETs-treated monocytes secreted more anti-inflammatory (TGF-β and IL-10) than pro-inflammatory (TNF-α and IL-6) cytokines. Accordingly, our data also show that NETs-treated monocytes presented a lower parasite killing capacity and generate M2 macrophages, suggesting that NETs might be activating an anti-inflammatory immune response in these cells.

Despite the ability of neutrophils to release traps in response to parasite presence, a portion of the population can escape NET-mediated killing. Parasites that have escaped NETs will infect other cells, such as inflammatory recruited monocytes, macrophages, and DCs. Upon reaching the site of infection, monocytes interact with NETs and parasites. We hypothesize
that NETs decrease the differentiation of monocytes into DCs and at the same time disables the microbicidal mechanisms of these monocytes, which differentiate into M2 macrophages. The data presented here provide new evidence about the role of NETs in Leishmania infection. The reduced expression of IL-4R caused by NETs may result in modulation of the immune response to the parasite and thereby alter the course of the disease. Furthermore, the effect in reducing differentiation of monocytes and decreasing the expression of IL-4 identified in this work can be applied to any other disease or inflammatory event where the interaction between neutrophils and monocytes occurs and is relevant. Hence, the relevance of the data presented here is not restricted to infection by the parasite Leishmania.

ETHICS STATEMENT

Human PBMCs from healthy subjects were obtained under written informed consent and all procedures were approved by the Institutional Review Board for Human Subjects (Research Ethics Committee) from Hospital Clementino Fraga Filho, Universidade Federal do Rio de Janeiro (protocol number 055-15) and from the NIH Clinical Center IRB-approved protocol from the NIH Clinical Center Department of Transfusion Medicine (protocol number 99-CC-0168).

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments and wrote the paper: AG-C, FO, JE-L, and ES. Performed the experiments: AG-C and NR. Analyzed the data: AG-C, NR, FO, JE-L, and ES.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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