

RESEARCH ARTICLE

Exposure to *Leishmania braziliensis* Triggers Neutrophil Activation and Apoptosis

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Abstract

Background

Neutrophils are the first line of defense against invading pathogens and are rapidly recruited to the sites of *Leishmania* inoculation. During *Leishmania braziliensis* infection, depletion of inflammatory cells significantly increases the parasite load whereas co-inoculation of neutrophils plus *L. braziliensis* had an opposite effect. Moreover, the co-culture of infected macrophages and neutrophils also induced parasite killing leading us to ask how neutrophils alone respond to an *L. braziliensis* exposure. Herein we focused on understanding the interaction between neutrophils and *L. braziliensis*, exploring cell activation and apoptotic fate.

Methods and Findings

Inoculation of serum-opsonized *L. braziliensis* promastigotes in mice induced neutrophil accumulation *in vivo*, peaking at 24 h. *In vitro*, exposure of thyoglycollate-elicited inflammatory or bone marrow neutrophils to *L. braziliensis* modulated the expression of surface molecules such as CD18 and CD62L, and induced the oxidative burst. Using mCherry-expressing *L. braziliensis*, we determined that such effects were mainly observed in infected and not in bystander cells. Neutrophil activation following contact with *L. braziliensis* was also confirmed by the release of TNF- α and neutrophil elastase. Lastly, neutrophils infected with *L. braziliensis* but not with *L. major* displayed markers of early apoptosis.

Conclusions

We show that *L. braziliensis* induces neutrophil recruitment *in vivo* and that neutrophils exposed to the parasite *in vitro* respond through activation and release of inflammatory mediators. This outcome may impact on parasite elimination, particularly at the early stages of infection.

Author Summary

Leishmania is the parasite responsible for the disease leishmaniasis, present in all continents. *Leishmania* parasites are spread through infected sand-flies and, during transmission into the vertebrate host, neutrophils are among the first cells to arrive at the infection site. Since neutrophils are key players at the frontline of defense against invading organisms, we investigated their response to *Leishmania braziliensis*. Importantly, *L. braziliensis* causes both Cutaneous and Mucocutaneous Leishmaniasis, two clinical manifestations characterized by their chronic development and by the presence of skin lesions with tissue destruction. Upon inoculation of mice with *L. braziliensis*, neutrophils rapidly arrive at the site of infection. We then observed that culture of mouse neutrophils with *L. braziliensis* induced the expression of adhesion molecules, production of Reactive Oxygen Species and secretion of elastase and TNF- α , two important inflammatory mediators. Also, infection with *L. braziliensis* induced neutrophil apoptosis, a cell death mechanism key for regulating inflammation. Our results show that neutrophils respond to presence of the *L. braziliensis* parasites by becoming activated and undergoing apoptosis. We suggest that this outcome modifies the local environment at the site of parasite inoculation and thus contributes with parasite killing in the infected host.

Introduction

Neutrophils are essential components of the early inflammatory response, acting as the first line of defense against invading pathogens (rev. in [1]). Neutrophil recruitment to the infection site occurs in response to various stimuli and is followed by cell rolling and adhesion to the vasculature, processes mediated by interactions between selectins and integrins [2]. Pathogen phagocytosis subsequently elicits the production of superoxide, which is quickly dismutated into hydrogen peroxide and other secondary Reactive Oxygen Species (ROS), which are highly toxic to the invading pathogen [3]. Phagocytosis stimulates the secretion of additional antimicrobial molecules such as neutrophil elastase, into the phagosome further contributing with pathogen killing [4]. Resolution of inflammation requires efficient removal of apoptotic neutrophils by professional phagocytes such as resident macrophages [5]. Phagocytosis of apoptotic neutrophils prevents the release of potentially toxic molecules and, in parallel, regulates the inflammatory response [6].

During experimental *Leishmania* infection, neutrophils play distinct roles depending on the combination of mouse strain and parasite species. For *L. donovani* and *L. infantum*, neutrophils contributed to parasite killing [7,8]. For *L. major*, neutrophil and monocyte depletion enhanced disease in resistant mice [9–12] whereas, in susceptible mice, the absence of neutrophils inhibited Th2 cell development [12]. Neutrophil depletion led to faster lesion development in mice infected with *L. amazonensis* promastigotes [13] whereas amastigotes displayed resistance to the neutrophil microbicidal machinery [14]. Following phagocytosis, some *Leishmania spp* can be found within non-lytic compartments [15]. This evasion strategy suggests that *Leishmania* parasites may exploit neutrophils as to gain access to macrophages where, ultimately, infection is established [16,17]. *In vivo*, neutrophils readily arrive at the site of *L. major* [12,18] and *L. infantum-chagasi* inoculation [19] within minutes. Employing a natural transmission model, Peters *et al.* showed that neutrophils capture *L. major* parasites at the site of sand fly bite, but the parasites remain viable [20]. In this model, the absence of neutrophils was unfavourable to infection. More recently, Ribeiro-Gomes *et al.* showed that in experimental infection, the route of inoculation (intradermal, subcutaneous or intraperitoneal) also

impacts on the capture of *L. major* parasites by neutrophils and on the establishment of infection [21].

Previously, we showed that *L. braziliensis*-infected macrophages co-cultured with live neutrophils display a reduced parasite load [22]. This outcome was dependent on the interaction between macrophages and neutrophils and was associated with the production of TNF α and superoxide. We suggested that clearance of neutrophils in *L. braziliensis*-infected mice promotes a pro-inflammatory environment, contributing with parasite clearance. Herein we investigated how exposure to *L. braziliensis* and internalization or not of the parasite impacts the neutrophil response.

Methods

Ethics statement

Female BALB/c mice, 6–8 weeks of age, were obtained from CPqGM/FIOCRUZ animal facility where they were maintained under pathogen-free conditions. All animal work was conducted according to the Guidelines for Animal Experimentation of the Colégio Brasileiro de Experimentação Animal and of the Conselho Nacional de Controle de Experimentação Animal. The local Ethics Committee on Animal Care and Utilization (CEUA) approved all procedures involving animals (L-03/2011).

Parasites

L. braziliensis promastigotes (strain MHOM/BR/01/BA788) [23] or transgenic *L. braziliensis* parasites expressing mCherry [24], kindly provided by Phillip Scott (University of Pennsylvania), were grown in Schneider's insect medium (LGC) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Parasite cultures were seeded at 10⁵ parasites/mL and were closely monitored to ascertain that parasites had reached the stationary phase (7 days). Before co-culture experiments with neutrophils, stationary-phase parasites were opsonized with 5% heat-inactivated fresh naïve serum for 30 min at 24°C. Metacyclic enriched promastigotes were obtained as described elsewhere [25]. In some experiments, we employed *L. major* (WHOM/IL/80/Friedlin) or dead parasites, prepared as described [26].

Neutrophil recruitment

BALB/c mice were inoculated in the ear dermis with 10⁶ stationary phase promastigotes, in 10 μ L, using a 27 1/2G needle. Control mice were injected with serum-free DMEM medium. After 6, 24 and 48h post-inoculation, mice were sacrificed and the dorsal and ventral ear sheets separated with forceps. The two leaflets were transferred to RPMI supplemented with 10% FCS and antibiotics. After 16h the cells emigrating out of the ear explants were collected, counted and stained for flow cytometry [27]. For cell surface molecules, mAb 24G2 was used to block FcRs and cells were stained using anti-Ly6G-APC/Cy7 (clone 1A8) (BioLegend) and anti-CD11b-eFluor 450 (clone M1/70) (eBioscience). All cell events were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star, Inc.).

Isolation of neutrophils

Peritoneal neutrophils (herein referred as inflammatory neutrophils) were obtained by i.p. injection of 10% thioglycollate (SIGMA), as described [28]. Cells were collected 18 h later, by peritoneal washings, counted, and were left to adhere for 1h at 37°C. Non-adherent cells were recovered, washed and examined for purity by both FACS and H&E staining of cytospin preparations. Neutrophils of purity >90% were used in experiments. Bone marrow neutrophils were

obtained from the tibia and femur of mice; labeled with neutrophil-specific mAbs anti-Ly6G (clone NIMP-R14-FITC or clone-1A8-PE) (BD Pharmingen) and purified by MACS-positive selection, using anti-FITC or anti-PE magnetic beads (Miltenyi Biotec). Alternatively, neutrophils were labeled with anti-Ly6G (clone 1A8, conjugated to Biotin, Miltenyi Biotec) and purified using anti-Biotin magnetic beads. Purity of neutrophils following either NIMP-R14 or 1A8 positive MACS selection was >95%, as assessed by FACS. Control stainings with CD11b and Ly6C were performed following magnetic separation and neutrophils (inflammatory and bone marrow) were characterized as CD11b⁺1A8⁺Ly6C^{int} and Gr1^{high} (S1 and S2 Figs, respectively).

Neutrophil culture with *L. braziliensis*

Inflammatory neutrophils or bone marrow neutrophils were cultured for 2h, in RPMI medium supplemented with 10% FCS, 100 U/ml of penicillin and 100 µg/ml of streptomycin (all from Invitrogen), in the presence or absence of serum-opsonized *L. braziliensis* (at a 2:1 parasite:cell ratio). The infection rate of inflammatory or bone marrow neutrophils co-cultured with *L. braziliensis*-expressing mCherry was determined by flow cytometry. Data were acquired on a Fortessa or an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star Inc.).

Expression of surface molecules and measurement of oxidative burst

Neutrophils were co-cultured *L. braziliensis*, as described, for 2 h. For cell surface staining, neutrophils were incubated with FcBlock (CD16/CD32) (BD Pharmingen) followed by anti-CD18-FITC (clone M18/2) or anti-CD62L-PE (clone MEL-14) (all from E-bioscience, including isotype control Rat IgG2a). For the detection of Reactive Oxygen Species, cells were co-cultured with *L. braziliensis* for 2 h and were later stained with Dihydroethidium (DHE) (Invitrogen), a superoxide indicator, at 3 µM for 30 minutes. As a positive control, neutrophils were incubated with phorbol 12-myristate 13-acetate (PMA) (100nM) (SIGMA) for 30 minutes. Data were acquired with a FACSAria or FACScan (BD Biosciences) and analyzed with FlowJo (Tree Star Inc.).

Measurement of Elastase activity and of TNF-α production

Neutrophils were co-cultured with *L. braziliensis*, as explained above, for 4h. Elastase enzymatic activity was measured as described [10]. Briefly, cell culture supernatants were harvested and added (20 µL) in triplicate to ELISA plates. Following addition of Elastase reaction buffer (55µL) (0.1 M HEPES, 0.5 M NaCl, 10% dimethylsulfoxide, pH 7.5) and 0.2 mM Elastase substrate I (MeOSuc-AAPV-pna; Calbiochem) (150 µL), samples were incubated at 37°C for 3 days. Elastase activity was determined by reading absorbance at 410 nm, using serial dilutions of human elastase (Calbiochem), as standards. For the detection of TNF-α, neutrophils were co-cultured with *L. braziliensis* for 24h. Cell culture supernatants were collected and TNF-α levels were determined by ELISA, using a commercial kit (R&D Systems).

Measurement of neutrophil apoptosis

Inflammatory neutrophils were co-cultured with *L. braziliensis* or *L. major* (at a 5:1 parasite:cell ratio) for 18 h. Neutrophils were then stained with Annexin V-FITC and PI (both from BD Biosciences). Bone marrow neutrophils were co-cultured with mCherry-*L. braziliensis*, as described above, for 18 h. Apoptotic neutrophils were obtained by ultraviolet irradiation exposure (245nm) for 10 minutes [10]. Cells were stained with Annexin V-FITC (Biolegend) and DAPI

(SIGMA) and apoptosis was assessed by flow cytometry. Data were acquired with a FACS Aria or FACScan (BD Biosciences) and analyzed with FlowJo (Tree Star, Inc.).

Transmission electron microscopy

Inflammatory neutrophils were co-cultured with *L. braziliensis* or *L. major* (at a 5:1 parasite: cell ratio) for 18 h. Cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and post-fixed in 1% OsO₄ and 0.8% potassium ferricyanide and 5 mM calcium chloride in the same buffer. Cells were dehydrated in a graded series of acetone and embedded in Poly/Bed 812 (Polysciences, Inc.) resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined on a Zeiss109 transmission electron microscope operating at 80 KV.

Statistical analysis

The significance of the results was calculated using non-parametrical statistical tests [Mann Whitney (two-sided t-test) or Kruskal-Wallis followed by Dunn's post test]. Analyses were conducted using Prism (GraphPad software) and a p-value of <0.05 was considered significant.

Results

Neutrophil recruitment to the site of *L. braziliensis* inoculation

Previously, we reported that neutrophils are present throughout the course of lesion development in BALB/c mice inoculated with *L. braziliensis* [22]. Herein, we initially evaluated the kinetics of neutrophil recruitment at the early moments following *L. braziliensis* infection. Mice were inoculated in the ear dermis with *L. braziliensis* parasites and recruited cells were selected based on size and granularity; within this population, we defined Ly6G⁺ neutrophils (Fig. 1A). Six hours following parasite inoculation, we did not see differences in the number of Ly6G⁺ neutrophils comparing mice inoculated with *L. braziliensis* and control mice, inoculated with saline (Fig. 1B). Twenty-four hours later, the number of Ly6G⁺ cells recruited to the inoculation site significantly increased in experimental mice and 48h later, this number decreased (p<0.05, compared to the 24 h time-point). These results show that neutrophil recruitment peaks one day after *L. braziliensis* inoculation.

Expression of CD62L and CD18 in *L. braziliensis*-exposed neutrophils

Following the observation that neutrophils rapidly accumulate at the site of *L. braziliensis* inoculation (Fig. 1), we investigated the expression of molecules important for cell rolling such as CD62L (L-selectin) and adherence and transmigration such as CD18 (β2 integrin). We also employed serum-opsonized *L. braziliensis* since *Leishmania* is delivered into the host in a blood pool, where promastigotes likely encounter serum and the complement system. Inflammatory neutrophils were co-cultured with serum-opsonized mCherry *L. braziliensis* and neutrophils were selected by size and granularity and, subsequently, by expression of Ly6G (Fig. 2A). In parallel, we also compared infected neutrophils (Ly6G⁺mCherry⁺) and bystander neutrophils (Ly6G⁺mCherry⁻), the latter defined as neutrophils that remained uninfected in spite of exposure to *L. braziliensis* (Fig. 2A). Following co-culture of inflammatory neutrophils with *L. braziliensis*, the percentage of infected (mCherry⁺) neutrophils was approximately 36% (Fig. 2B) whereas 49% of cells remained uninfected (mCherry⁻). The percentage of CD18⁺ cells among infected (mCherry⁺) neutrophils was higher (p<0.01) in comparison to bystanders (mCherry⁻) (Fig. 2B) whereas in control cultures (not exposed to neutrophils) the percentage of CD18⁺ cells was very low (Fig. 2B). In non-exposed neutrophils, the percentage of CD62L⁺ cells was

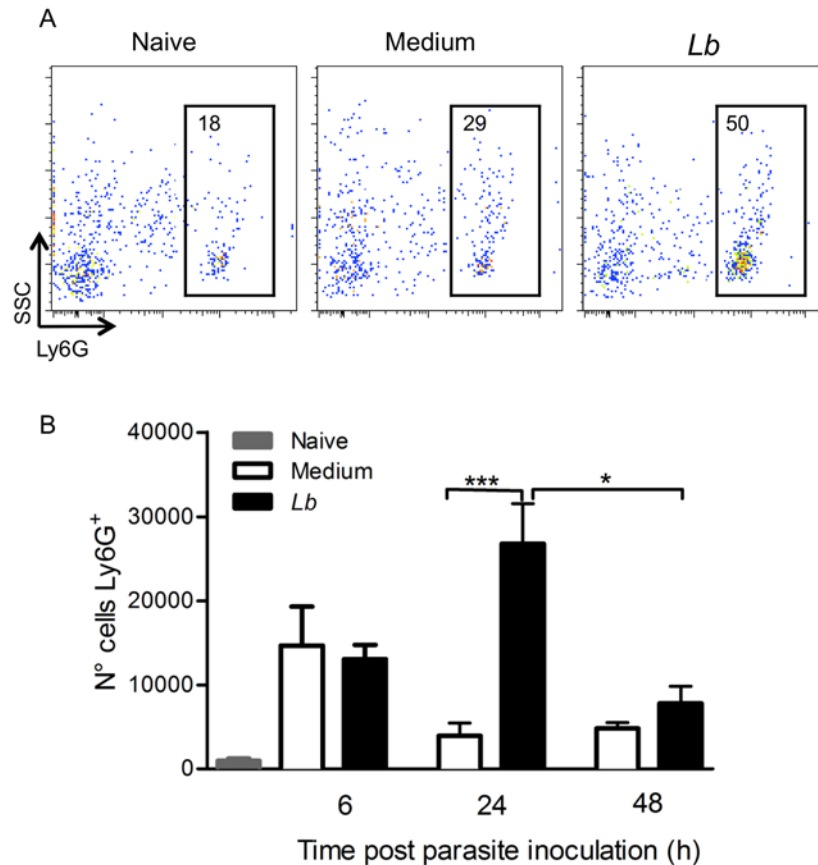


Fig 1. Neutrophil recruitment following *L. braziliensis* inoculation. Individual BALB/c mice were injected with *L. braziliensis* in the ear dermis. At different time points, cells were prepared and stained for Ly6G and analyzed by flow cytometry. (A) Representative dot plots and gating strategy of neutrophils. (B) Bar graph representation of the number of Ly6G⁺ cells in naïve mice (grey bars), in mice inoculated with saline (white bars) or inoculated with *L. braziliensis* (black bars). Data shown (mean ± SEM) are from one experiment representative of two. ***p < 0.001, *p < 0.05.

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high (Fig. 2C) and differently from CD18, the percentage of CD62L⁺ cells was lower (p < 0.05) among infected (mCherry⁺) neutrophils compared to bystanders (mCherry⁻) (Fig. 2C). Incubation of inflammatory neutrophils with Zymozan did not significantly alter the percentage of CD18⁺ cells (S3 Fig).

To expand on these findings, we performed experiments with bone marrow neutrophils, which, comparatively have an enhanced capacity to become primed [29]. Bone-marrow neutrophils were also selected by size, granularity and Ly6G expression (Fig. 3A) and following co-culture with *L. braziliensis*, the percentage of mCherry⁺ neutrophils was approximately 48% (Fig. 3A) whereas 43% of cells were mCherry⁻. As with inflammatory neutrophils (Fig. 2), the percentage of CD18⁺ cells was also very low in control non-exposed cultures and significantly higher (p < 0.05) among infected (mCherry⁺) neutrophils compared to bystanders (mCherry⁻) (Fig. 3B). Also replicating our findings with inflammatory neutrophils (Fig. 2), the percentage of CD62L⁺ cells was highest in non-exposed neutrophils (Fig. 3C), and significantly higher (p < 0.05) in bystanders (mCherry⁻) compared to infected (mCherry⁺) neutrophils. These data indicate that neutrophils infected with *L. braziliensis* upregulate CD18 and downregulate CD62L, regardless of their activation state.

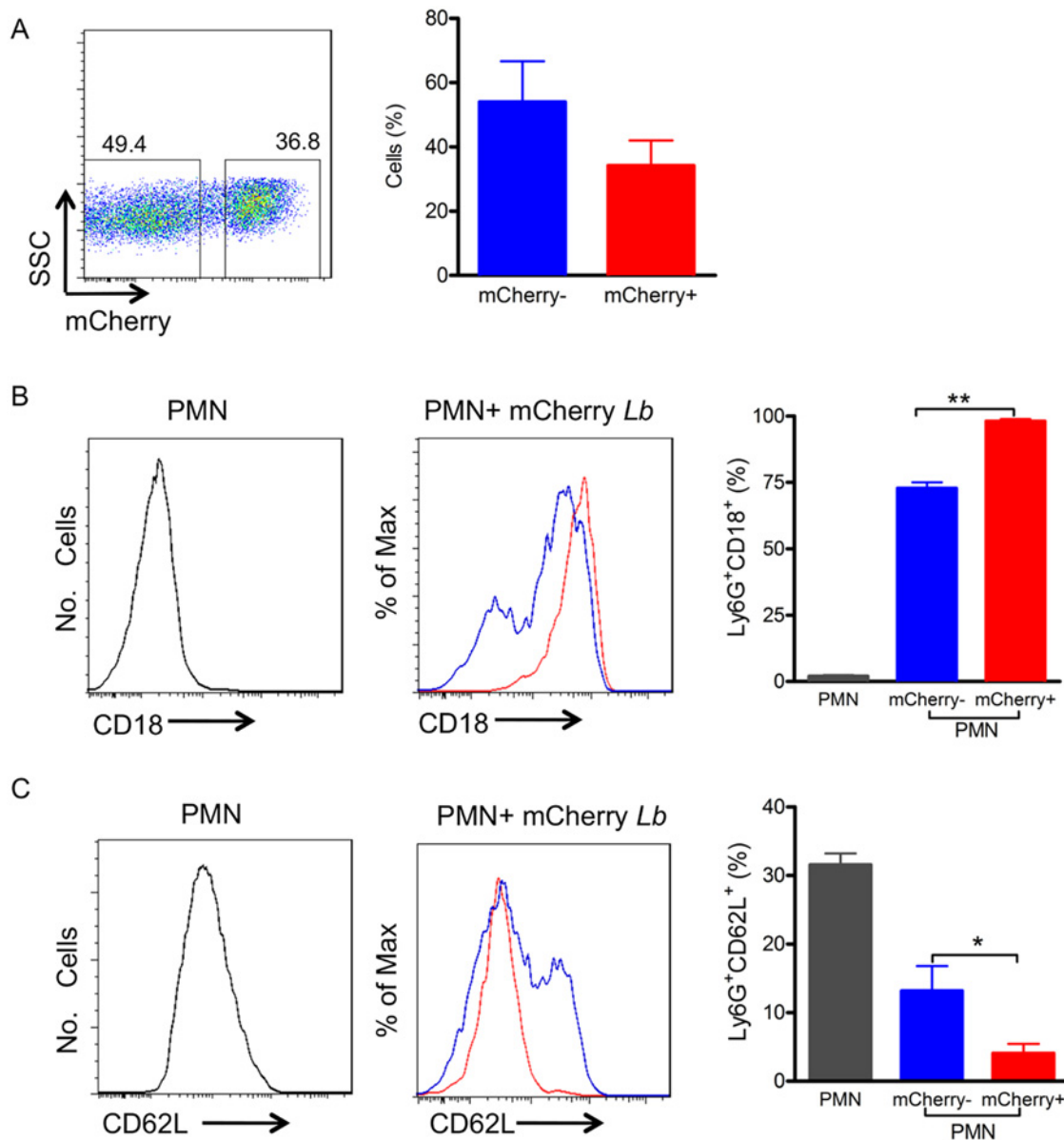


Fig 2. Inflammatory neutrophils infected with *L. braziliensis* up-regulate CD18 and down-regulate CD62L. Inflammatory neutrophils were co-cultured with mCherry *L. braziliensis*. (A) Representative dot plot and bar graph depicting the percentage of non-infected (mCherry⁻) and infected (mCherry⁺) neutrophils. Representative histograms depicting CD18 (B) and CD62L (C) staining in control neutrophils cultured alone (gray), bystander neutrophils (mCherry⁻) (blue, shown as % of Max) and infected neutrophils (mCherry⁺) (red, shown as % of Max). Bar graphs represent the percentages of control, bystander and infected neutrophils expressing CD18 (B) and CD62L (C). Data shown (mean ± SEM) are pooled from two independent repeats. **p<0.01; *p<0.05.

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Production of superoxide in neutrophils exposed to *L. braziliensis*

Neutrophils produce Reactive Oxygen Species (ROS), which form a central component of the defense mechanism against foreign pathogens during infection. Inflammatory neutrophils co-cultured with *L. braziliensis* displayed a significant increase in superoxide production (Fig. 4A), which was attributed mostly to neutrophils harboring *L. braziliensis*-mCherry. With bone marrow neutrophils, superoxide production was also significantly higher in cells harboring mCherry, however, ROS was also observed in bystanders (mCherry⁻) (Fig. 4B). Additionally,

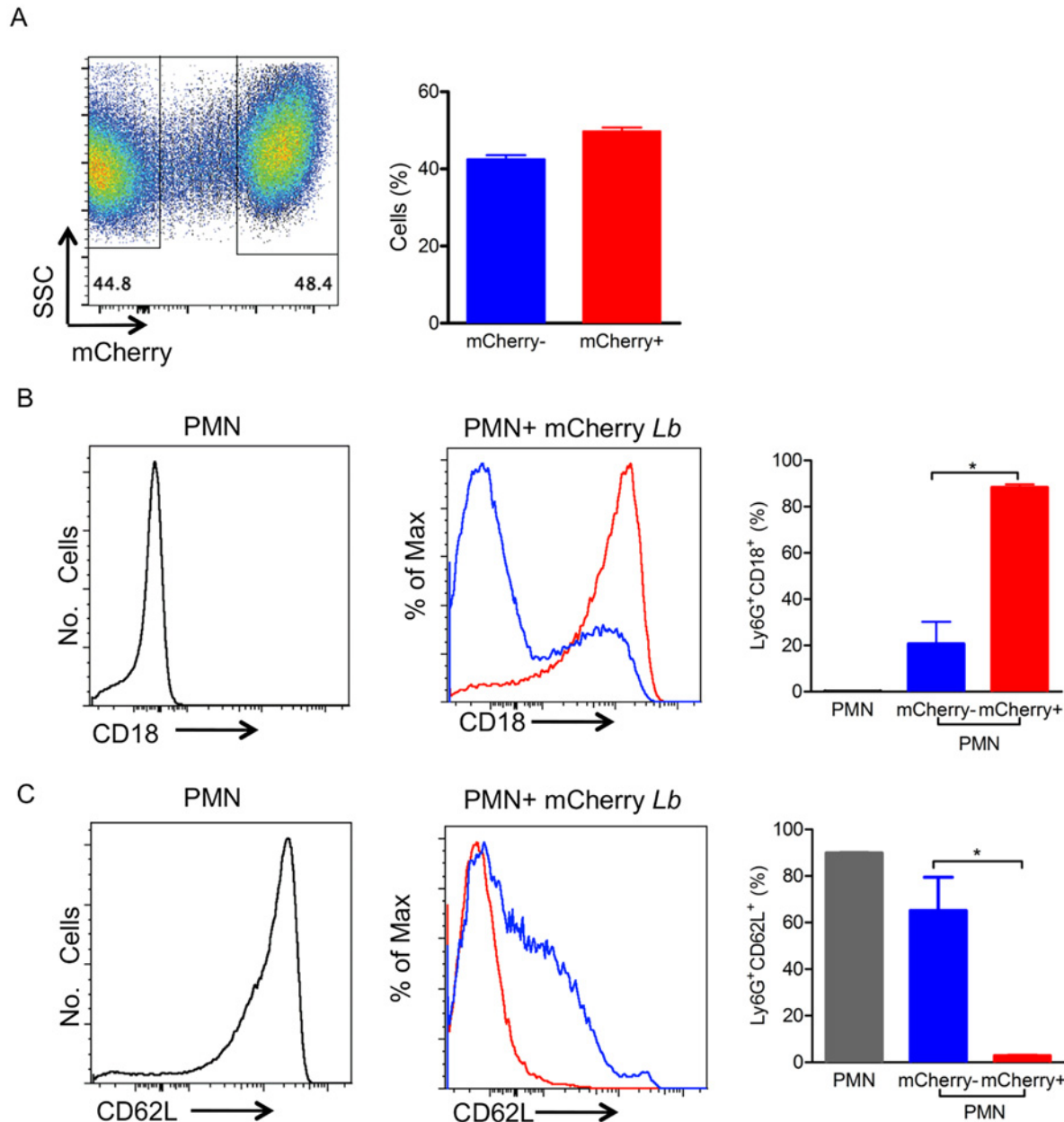


Fig 3. Bone-marrow neutrophils infected with *L. braziliensis* up-regulate CD18 and down-regulate CD62L. Bone-marrow neutrophils were co-cultured with mCherry *L. braziliensis*. (A) Representative dot plot and bar graph representing the percentage of non-infected (mCherry⁻) and infected (mCherry⁺) neutrophils. Representative histograms depicting CD18 (B) and CD62L (C) staining in control neutrophils cultured alone (gray), bystander neutrophils (mCherry⁻) (blue, shown as % of Max) and infected neutrophils (mCherry⁺) (red, shown as % of Max). Bar graphs represent the percentages of control, bystander and infected neutrophils expressing CD18 (B) and CD62L (C). Data shown (mean ± SEM) are pooled from two independent repeats. *p < 0.05.

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superoxide production was similar upon co-culture of inflammatory neutrophils with either stationary phase or metacyclic *L. braziliensis* (Fig. 5A and B). Co-culture with dead parasites also did not change superoxide production in relation to neutrophils cultured alone (Fig. 5A and B). Similar results were obtained regarding the percentage of CD18⁺ cells (Fig. 5C and D).

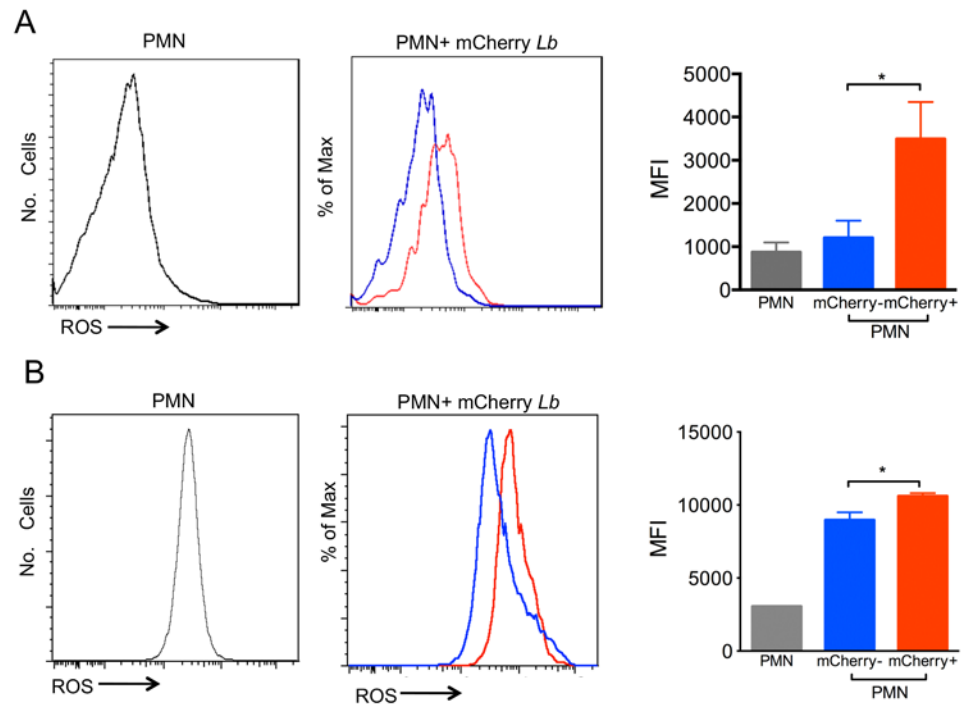


Fig 4. Exposure to *L. braziliensis* induces ROS production. Neutrophils were co-cultured with mCherry *L. braziliensis*, cells were stained with DHE and ROS production was analyzed by FACS. (A) Histograms represent inflammatory neutrophils cultured alone (gray), bystander neutrophils (mCherry⁻) (blue) and infected neutrophils (mCherry⁺) (red). (B) Histograms represent bone marrow neutrophils cultured alone (gray), bystander neutrophils (mCherry⁻) (blue) and infected neutrophils (mCherry⁺). Bar graphs represent the MFI of neutrophils cultured alone (PMN), bystanders (mCherry⁻) (blue) or *L. braziliensis*-infected (mCherry⁺) (red). Data (mean ± SEM) are from one experiment representative of two. *p<0.05.

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Production of TNF- α and elastase by neutrophils exposed to *L. braziliensis*

Neutrophils display granules enriched with antimicrobial molecules, including serine proteases such as elastase [30]. Additionally, cytokines secreted by neutrophils, such as TNF- α , influence macrophage and dendritic cell function, with important effects on the adaptive immune response [28]. Herein, co-culture with *L. braziliensis*, triggered the release of elastase by both inflammatory (Fig. 6A) and bone marrow neutrophils (Fig. 6B). In the same manner, the presence of TNF- α was significantly higher in cultures of inflammatory (Fig. 6C) and bone marrow neutrophils (Fig. 6D) co-cultured with *L. braziliensis*. We did not detect IL-10 nor IL-12p40 in the culture supernatants.

Neutrophil apoptosis following exposure to *L. braziliensis*

At infection sites, cells dying by apoptosis express phosphatidylserine (PS) and PS exposure can be detected by Annexin V staining and quantified by flow cytometry. Inflammatory neutrophils were co-cultured for 18 h with *L. braziliensis* parasites and we investigated whether this interaction resulted in apoptosis. In these co-cultures, there was a significant (p<0.05) increase in the percentage of early apoptotic (Annexin V⁺/PI⁻) neutrophils (Fig. 7A and B), compared to neutrophils cultured alone, whereas the percentage of late apoptotic/necrotic (Annexin V⁺/PI⁺) neutrophils was similar (Fig. 7A and B). On the other hand, upon co-culture with *L. major*, we detected a lower percentage of both early (Annexin V⁺/PI⁻) and late

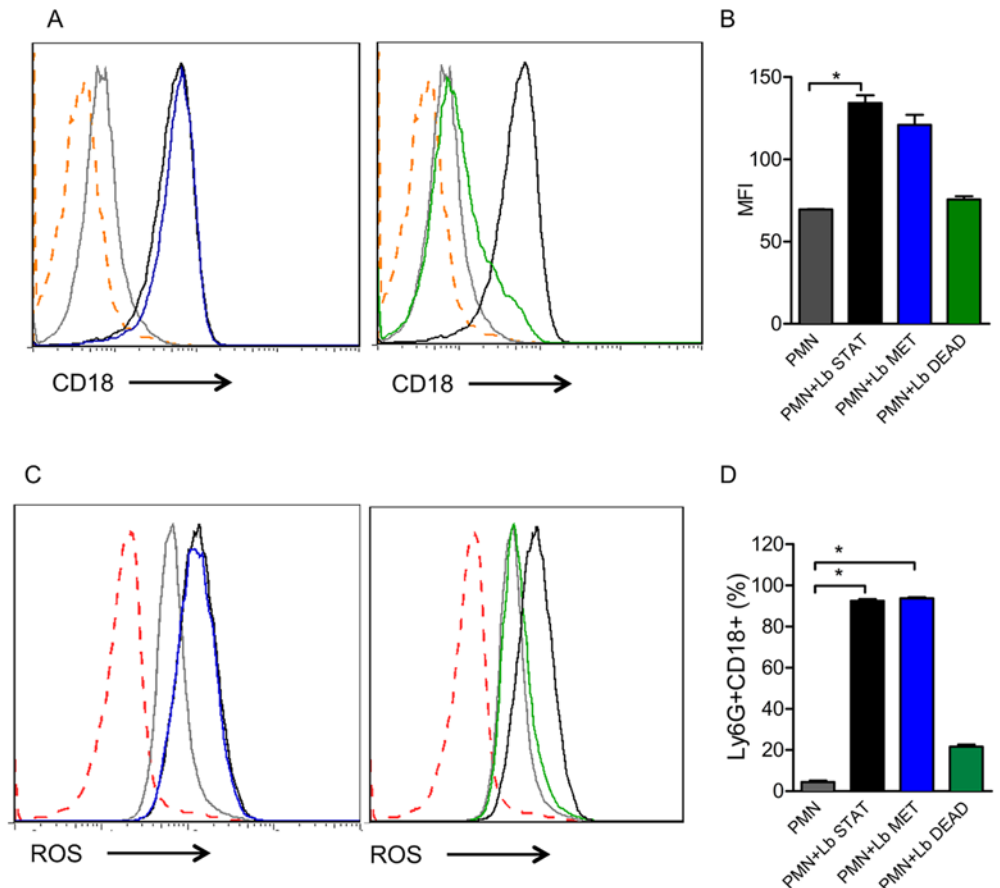


Fig 5. Exposure to dead *L. braziliensis* does not modulate CD18 expression or ROS production. Inflammatory neutrophils were co-cultured with *L. braziliensis* and stained with anti-CD62L. (A, C) Representative histograms depicting ROS production or CD18 staining in neutrophils cultured alone (gray), neutrophils exposed to stationary *L. braziliensis* (black), metacyclic *L. braziliensis* (blue) or dead *L. braziliensis* (green). Dotted orange line depicts unstained neutrophils. (B, D) Bar graphs represent the percentage of neutrophils positive for CD18. Data shown (mean \pm SEM) are from one experiment representative of two. * $p < 0.05$.

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apoptotic/necrotic (Annexin V⁺/PI⁺) neutrophils, compared to neutrophils cultured with *L. braziliensis* (Fig. 7A and B). Analysis of neutrophils by transmission electron microscopy confirmed apoptosis of *L. braziliensis*-infected neutrophils as seen by the presence of pyknosis, chromatin condensation as well as remnants of internalized degenerated parasites (Fig. 8). Moreover, internalized *L. braziliensis* parasites presented chromatin condensation, cytoplasmic disorganization and vacuolization (Fig. 8). In co-cultures performed with *L. major*, however, the neutrophils remained with a well preserved cytoplasm and viable parasites were observed inside the parasitophorous vacuole (Fig. 8), reinforcing the finding that *L. major* delays neutrophil apoptosis [31], differently from *L. braziliensis*.

Following the observation that *L. braziliensis* induced apoptosis in inflammatory neutrophils, we then examined whether this would also occur with bone marrow neutrophils. Indeed, upon co-culture with *L. braziliensis*, a significant increase in the percentage of late apoptotic/necrotic (Annexin V⁺/DAPI⁺) neutrophils (Fig. 9A and B) was observed. As a control of late apoptosis/necrosis, neutrophil exposure to UV increased the percentage of cells positive for Annexin V⁺/DAPI⁺. Importantly, late apoptosis/necrosis (Annexin V⁺/DAPI⁺) was mostly

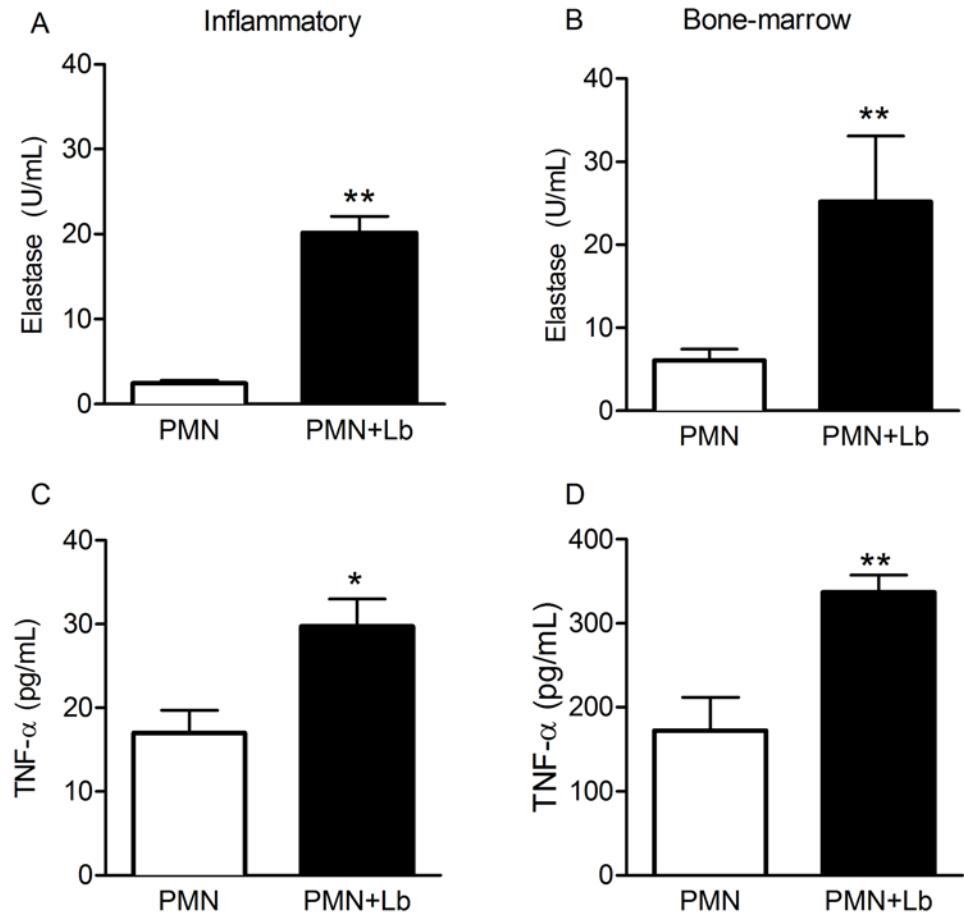


Fig 6. Production of Elastase and TNF- α by *L. braziliensis*-exposed neutrophils. Inflammatory or bone marrow neutrophils were co-cultured with *L. braziliensis* for 24h. Culture supernatants were assayed for the presence of TNF- α (A,B) and for the presence of free elastase activity (C,D). Data shown (mean \pm SEM) are pooled from two independent repeats. **p < 0.01, *p < 0.05.

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detected in infected neutrophils (mCherry⁺) when compared with bystanders (mCherry⁻) (Fig. 9C). At this time point, the percentage of mCherry⁺ neutrophils was ~28% (Fig. 9D).

Discussion

Numerous studies have demonstrated that neutrophils play a crucial role in immunity against bacterial, fungal [1] and intracellular pathogens [32]. Earlier on, we demonstrated that *L. braziliensis* inoculation into the ear dermis of BALB/c mice leads to the development of a cutaneous ulcer, which heals spontaneously after ten weeks of infection [23]. Additionally, co-inoculation of *L. braziliensis* and neutrophils decreased lesion size whereas depletion of neutrophils and monocytes had an opposing effect, significantly increasing parasite load and lesion size [22]. Given that neutrophils are among the first cells to encounter the parasite at the site of the sand fly bite [20] and, thus, will readily encounter *Leishmania* parasites, the purpose of the current study was to investigate how neutrophils respond to *L. braziliensis* exposure, evaluating neutrophil activation and downstream events such as apoptosis.

Sand flies probe the human host to obtain blood and, in this process, lacerate capillaries forming a blood pool into which *Leishmania* promastigotes are inoculated. Following this

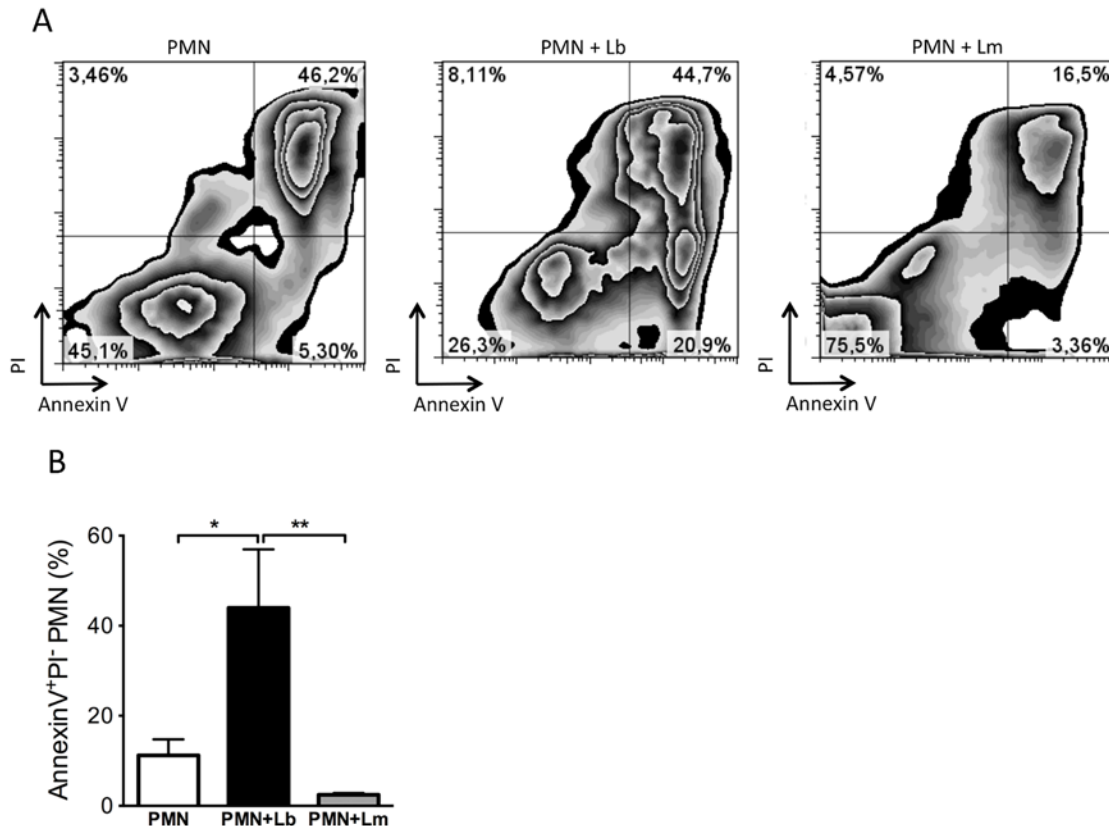


Fig 7. Exposure to *L. braziliensis* but not to *L. major* triggers neutrophils apoptosis. Inflammatory neutrophils were co-cultured with *L. braziliensis*. After 18h, neutrophils were labeled with Annexin V and PI and analyzed by FACS. (A) Representative zebra plots of Annexin V⁺/PI⁺ neutrophils (PMN) and of neutrophils co-cultured with *L. braziliensis* (PMN+Lb) or with *L. major* (PMN+Lm). (B) Percentage of Annexin V⁺/PI⁻ neutrophils alone (PMN) or neutrophils cultured with parasites (PMN+Lb and PMN+Lm). Data shown (mean ± SEM) are pooled from two independent repeats. *p<0.05; **p<0.01.

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event, there is rapid accumulation of neutrophils [20] and it has been shown that the co-inoculated salivary molecules can modulate neutrophil function [33,34]. Herein, we confirmed neutrophil infiltration to the site of *L. braziliensis* inoculation by syringe and showed maximal accumulation at 24h. Of interest, syringe inoculation of *L. amazonensis*, also induced maximal neutrophil accumulation at 24 h [13], indicating a common kinetic for neutrophil recruitment for these two New World *Leishmania* species.

Following our observation that neutrophils are recruited in response to *L. braziliensis* inoculation, we then performed a series of *in vitro* experiments to investigate how neutrophils respond to this type of stimulation and, in addition, we compared the responses of inflammatory and bone marrow neutrophils. Initially, we evaluated the expression of adhesion molecules. β_2 integrins are leukocyte-specific integrins required for neutrophil adhesion and transmigration across the activated endothelium [35] and CD18 is the common β_2 integrin present in LFA1 (CD11aCD18), Mac-1/CR3 (CD11bCD18) and p150/94/CR4 (CD11cCD18). In the presence of *L. braziliensis* we detected an increase in the percentage of neutrophils (inflammatory and bone marrow) expressing CD18 and this increase was associated with infected neutrophils (mCherry⁺), indicating that *L. braziliensis* were readily internalized. Indeed, Mac-1/CR3 (CD11bCD18) plays a major role in the phagocytosis of complement-opsonized *L. major* promastigotes by both macrophages [36–38] and human neutrophils [39].

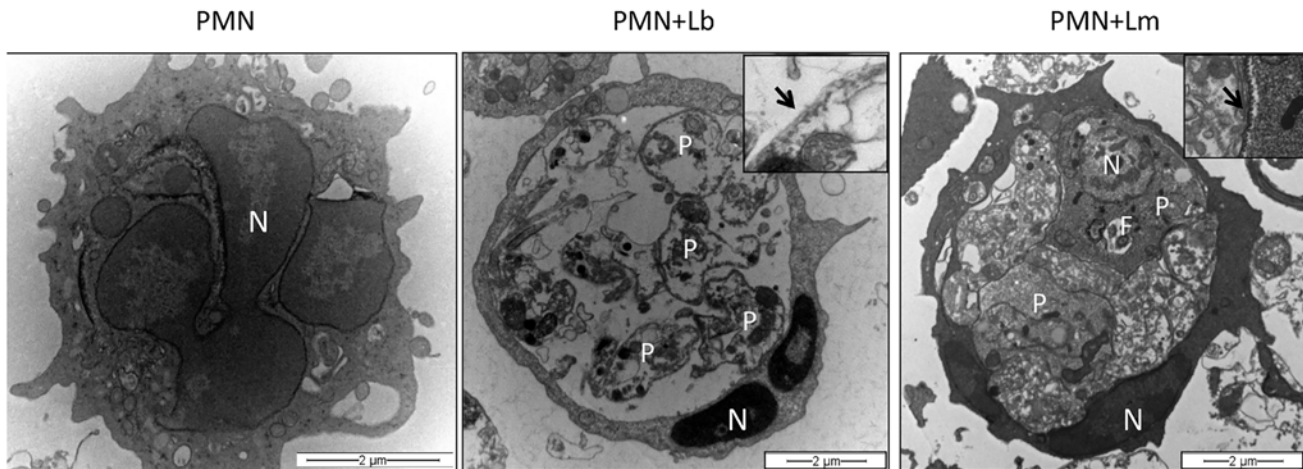


Fig 8. Ultrastructural analysis of neutrophils infected with *L. braziliensis* or *L. major*. Inflammatory neutrophils were co-cultured with *L. braziliensis* (at a 5:1 parasite:cell ratio), for 18 h. Cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and post-fixed in 1% OsO₄ and 0.8% potassium ferricyanide and 5 mM calcium chloride in the same buffer. Cells were dehydrated in a graded series of acetone and embedded in Poly/Bed 812 (Polysciences, Inc.) resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined on a Zeiss109 transmission electron microscope operating at 80 KV. (A) Uninfected neutrophils exhibiting multilobular nucleus (N). (B) Neutrophil infected with *L. braziliensis* showing condensed nucleus (N) and unpreserved intracellular parasite structures (P). Insert shows *Leishmania* microtubule (arrow). (C) Neutrophil infected with *L. major* showing preserved parasite structures (P) and nucleus (N). Insert shows *Leishmania* microtubule (arrow).

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L-selectin (CD62L) participates in neutrophil tethering and rolling [40] but it is cleaved from the leukocyte surface following cellular activation and exposure to inflammatory stimuli [41,42]. Upon co-culture with *L. braziliensis*, we detected a lower percentage of inflammatory CD62L⁺/mCherry⁺ neutrophils, compared to bystanders (mCherry⁻). Similar results were obtained with bone marrow neutrophils, indicating that *L. braziliensis* phagocytosis induced more CD62L shedding, marking neutrophil activation [43]. With regards to bystanders, the percentage of inflammatory CD62L⁺/mCherry⁻ neutrophils was lower compared to bone marrow (CD62L⁺/mCherry⁻) neutrophils, possibly reflecting their already primed nature and their extravasation to the peritoneum following thyoglycollate stimulation. Such difference may also be related to the priming potential of bone marrow neutrophils vs. inflammatory, as shown by fMLP stimulation and induction of ROS [29]. Also, we cannot presently attribute CD62L shedding to the infection rate since mCherry staining was similar for both bone marrow (~48%) and inflammatory (~36%) neutrophils. Although we do not know which molecules may be activating bystander neutrophils, it has been shown that *L. amazonensis* LPG activates human neutrophils in levels similar to those observed with promastigotes [44].

In the presence of *L. braziliensis*, both inflammatory and bone marrow neutrophils displayed a significant increase in the production of superoxide, a hallmark of neutrophil activation, and ROS detection was significantly higher in infected (mCherry⁺) neutrophils. Similar results were obtained in experiments with other *Leishmania* spp. [14,44–46]. Neutrophils exposed to ROS also up-regulate the production of TNF- α and MIP-2 [47,48] and TNF- α primes murine neutrophils to become activated, an effect that is concomitant with the mobilization of CR3-containing granules to the plasma membrane [49]. Since TNF- α and CD18 expression were increased upon neutrophil-co-culture with *L. braziliensis*, we can suggest that ROS produced by infected cells contributed with TNF- α secretion and CD18 (a Mac1/CR3 component) expression. Furthermore, elastase production was also elevated in neutrophils cultured with *L. braziliensis* and, importantly, elastase was associated with the killing of intracellular *Leishmania* in macrophages cultured with neutrophils [10], a process dependent on TLR4 signaling

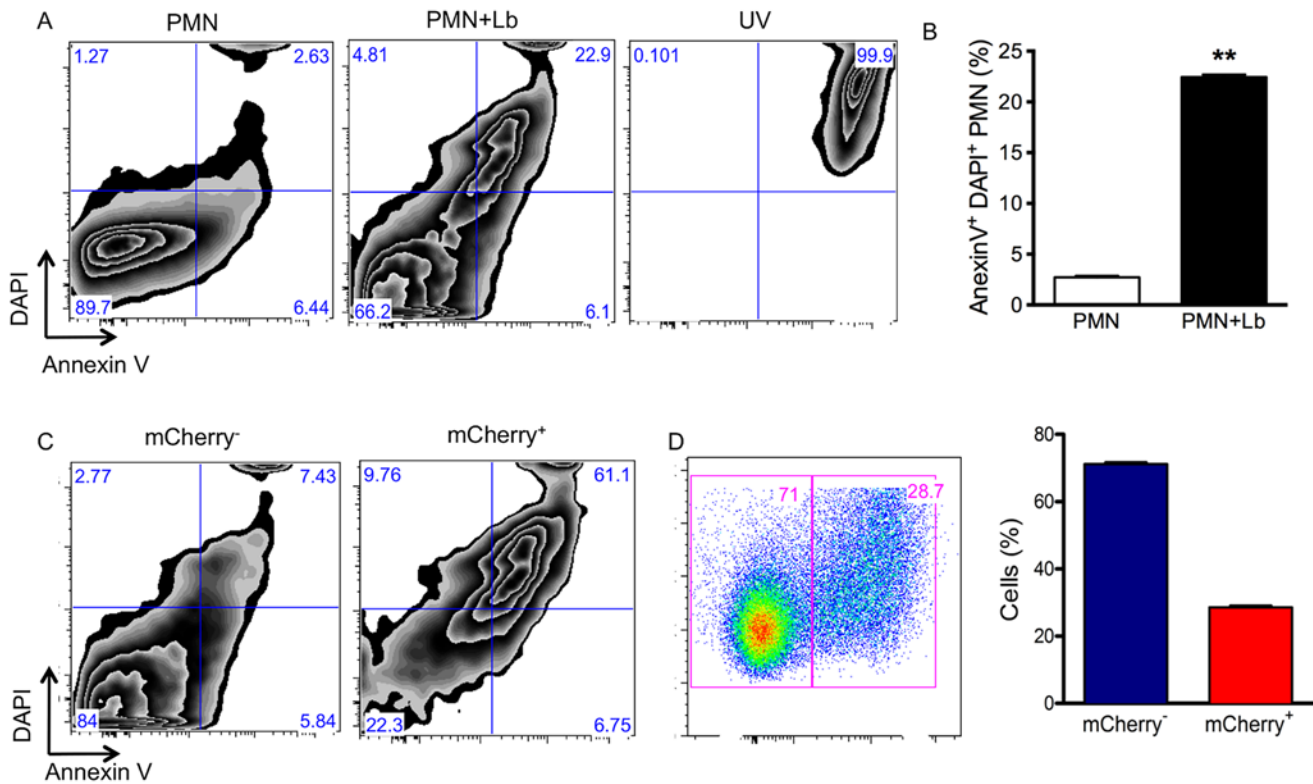


Fig 9. Apoptosis in neutrophils infected with *L. braziliensis*. Bone-marrow neutrophils were co-cultured with *L. braziliensis*. After 18h, neutrophils were labeled with Annexin V and DAPI and analyzed by FACS. (A) Representative zebra plots of neutrophils (PMN), neutrophils cultured with *L. braziliensis* (PMN+Lb) or neutrophils exposed to UV (UV). (B) Percentage of DAPI⁺/Annexin V⁺ neutrophils (PMN) or neutrophils cultivated with *L. braziliensis* (PMN+Lb). (C) Representative zebra plots of bystander neutrophils (mCherry⁻) and of infected neutrophils (mCherry⁺) expressing DAPI and Annexin V. (D) Representative dot plots of neutrophils infected with mCherry *L. braziliensis* after 18h, bar graph represents the percentage of non-infected (mCherry⁻) and infected (mCherry⁺) neutrophils. Data (mean ± SEM) are from one experiment representative of three experiments. **p < 0.01.

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[50]. IL-10 production, on the other hand, was not modulated in our experiments, as seen in previous studies [14,51].

Cell death and the subsequent clearance of apoptotic neutrophils is crucial for maintaining homeostasis and, at the same time, necessary for resolution of inflammation. At inflammatory sites, neutrophils can undergo spontaneous apoptosis [52] or apoptosis due to the recognition of cell-death mediators such as TNF- α and FasL [53]. Co-culture with *L. braziliensis* induced neutrophil apoptosis, findings that were confirmed by transmission electron microscopy analysis. Indeed, infected neutrophils displayed condensed chromatin and degraded intracellular parasites. Similar results were obtained with bone marrow neutrophils: Annexin⁺/DAPI⁺ staining was significantly higher in infected cells (mCherry⁺) compared to bystanders (mCherry⁻) and the percentage of infected neutrophils (mCherry⁺) was lower compared to bystanders (mCherry⁻). We can suggest that phagocytosis of *L. braziliensis* results in apoptosis and, in parallel, parasite destruction, hence the lower percentage of infected cells. In addition, ROS [54] and TNF- α [55] also trigger neutrophil apoptosis, two mediators that were produced upon culture with *L. braziliensis*.

Neutrophil apoptosis was also observed upon culture of neutrophils with *L. amazonensis* [14] but *L. major*, on the other hand, delays neutrophil apoptosis [31], enhancing cell lifespan [56]. Parasites survive within infected neutrophils [39,57] and viable parasites have been recovered by cell sorting [20]. Indeed, in our hands, the frequency of late apoptotic (Annexin⁺/PI⁺)

staining was low in neutrophils cultured with *L. major* in contrast to neutrophils cultured alone and to neutrophils cultured with *L. braziliensis*, both of which were positive for Annexin/PI (Fig. 6). Electron microscopy confirmed the presence remnants of *L. braziliensis* parasites while in contrast, intact parasites were found within *L. major* infected neutrophils (Fig. 7). Clearance of apoptotic neutrophils by macrophages promotes parasite replication *in vitro* [58], indicating that *L. major* may exploit neutrophil apoptosis as means to ascertain infection. Moreover, the phagocytosis of apoptotic neutrophils inhibits the response to *L. major* [59]. Therefore, for *L. major*, current literature indicates that neutrophils are rapidly and massively recruited to the site of *Leishmania* inoculation, where they phagocytose the parasites. Depending on the source of neutrophils, species and strains of *Leishmania*, internalized parasites can survive and neutrophils would thus provide a transient safe shelter prior to parasite entry into macrophages, the definitive host cell (rev. in [17,60]). In experiments with *L. braziliensis*, however, co-culture of infected macrophages with UV-treated neutrophils did not modulate the parasite load [22], also suggesting that differences within *Leishmania* species may induce distinct outcomes regarding neutrophil apoptosis and downstream effects.

We showed that neutrophils are recruited to the site of *L. braziliensis* inoculation and upon contact with promastigotes, *in vitro*, neutrophils become activated producing superoxide, TNF- α and elastase. Later, we observed neutrophil apoptosis, particularly of infected cells. However, once amastigotes become predominant, a different scenario may ensue since this stage is more resistant to these same effector mechanisms, as recently described for *L. amazonensis* [14], impacting on disease development. Indeed, BALB/c mice infected with *L. braziliensis* develop cutaneous ulcers, despite the presence of neutrophils [23]. However, in this experimental model lesions heal spontaneously and parasites are eliminated from the infection site. Neutrophils could also play a role at the chronic stages of infection, through cooperation with *L. braziliensis*-infected macrophages, as previously shown *in vitro* [22]. Neutrophils have been shown to cross-talk with dendritic cells [27,61,62] and such cross talk may also be related to the development of the adaptive immune response to *L. braziliensis*. However this remains to be investigated. Thus, the strong impact of *L. braziliensis* on neutrophils phenotype and function reported here *in vitro* are likely to occur at the onset of infection with the parasite, suggesting that these cells are playing a crucial role following infection.

Supporting Information

S1 Fig. Characterization of inflammatory neutrophils by flow cytometry. Inflammatory neutrophils were purified using MACS and the 1A8 (Ly6G) mAb. In this sample cells were first gated for size and granularity (SSC x FSC). The gated cells were further analyzed for expression of CD11b/1A8 or 1A8/Ly6C.
(TIF)

S2 Fig. Characterization of bone marrow neutrophils by flow cytometry. Bone marrow neutrophils were purified using MACS and the 1A8 (Ly6G) mAb. In this sample cells were first gated for size and granularity (SSC x FSC). The gated cells were further analyzed for expression of CD11b/1A8 or Gr-1/Ly6C.
(TIF)

S3 Fig. Modulation in CD18 expression is associated with *L. braziliensis* exposure. Inflammatory neutrophils were co-cultured with *L. braziliensis* or with Zymozan (SIGMA (100ug/ml)). (A) Representative dot plots showing the gating strategy used to identify neutrophils (Ly6G) following exposure to *L. braziliensis*. Cells were stained with anti-CD18 (B) and were analyzed by FACS. Orange histograms: isotype control. Gray histogram: neutrophils cultured

in medium only. Red histograms: neutrophils cultured with Zymozan. Black histograms, neutrophils exposed to *L. braziliensis*. Bar graphs represent the percentage of neutrophils positive for CD18. Data shown (mean \pm SEM) are pooled from two independent repeats. * $p < 0.05$ (One Way ANOVA). (TIF)

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Author Contributions

Conceived and designed the experiments: SACF TW BPH VMB FTC CIdO. Performed the experiments: SACF TW BPH FSC RPC DBP. Analyzed the data: SACF TW BPH DBP FSC RPC VMB FTC CIdO. Contributed reagents/materials/analysis tools: AB. Wrote the paper: SACF TW DBP VMB FTC CIdO.

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