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Degranulating Neutrophils Promote Leukotriene B₄ Production by Infected Macrophages To Kill Leishmania amazonensis Parasites

Natâlia Tavares,*¹ Lilian Afonso,*¹ Martha Suarez,*† Mariana Ampuero,*† Deboraci Brito Prates,*† Théo Araújo-Santos,*² Manoel Barral-Netto,*†,*‡ George A. DosReis,*§,† Valéria Matos Borges,*†,*³ and Cláudia Brodskyn*†,*³

Neutrophils mediate early responses against pathogens, and they become activated during endothelial transmigration toward the inflammatory site. In the current study, human neutrophils were activated in vitro with immobilized extracellular matrix proteins, such as fibronectin (FN), collagen, and laminin. Neutrophil activation by FN, but not other extracellular matrix proteins, induces the release of the granules’ contents, measured as matrix metalloproteinase 9 and neutrophil elastase activity in culture supernatant, as well as reactive oxygen species production. Upon contact with Leishmania amazonensis–infected macrophages, these FN-activated neutrophils reduce the parasite burden through a mechanism independent of cell contact. The release of granule proteases, such as myeloperoxidase, neutrophil elastase, and matrix metalloproteinase 9, activates macrophages through TLRs, leading to the production of inflammatory mediators, TNF-α and leukotriene B₄ (LTB₄), which are involved in parasite killing by infected macrophages. The pharmacological inhibition of degranulation reverted this effect, abolishing LTB₄ and TNF production. Together, these results suggest that FN-driven degranulation of neutrophils induces the production of LTB₄ and TNF by infected macrophages, leading to the control of Leishmania infection. The Journal of Immunology, 2016, 196: 1865–1873.

Leishmaniasis is a complex of diseases that is endemic to 90 countries, where 2 million new cases are reported each year (1). The protozoan parasite Leishmania is the etiologic agent of this disease (2). In the New World, the cutaneous forms are caused by Leishmania mexicana complex. Localized cutaneous leishmaniasis is the most frequent form of this disease and can be caused by L. braziliensis and L. amazonensis. However, L. amazonensis can also cause a severe form of the disease, known as diffuse cutaneous leishmaniasis, which is characterized by multiple nodules.

During Leishmania transmission, neutrophils are the first lineage of phagocytes recruited and infected at early stages in the skin, and they play important roles in the immunopathogenesis of the disease (3, 4). Intravital imaging was used to provide a visual demonstration of the rapidity of neutrophil migration to the infection site (5). Neutrophils appear to take up parasites at the early steps of infection and deliver the parasites to macrophages at a later stage. Additionally, we recently demonstrated the ability of human neutrophils to kill L. amazonensis promastigotes in vitro (6). We showed that exposure to promastigotes induces neutrophil activation as early as 30 min after infection. These cells also degranulate in a manner that is dependent on leukotriene B₄ (LTB₄) production and NF-kB activation. LTB₄ is a proinflammatory lipid mediator that is generated by 5-lipoxygenase (5-LO) and enhances the antimicrobial effector functions and cytokine production of macrophages (7, 8).

During migration from the bloodstream to the inflammatory site, neutrophils cross the vascular endothelium barrier, interacting with extracellular matrix (ECM) proteins; they become activated during this process (9). Neutrophils treated with ECM proteins show increased expression of activation surface markers, as well as degranulation and cytokine and chemokine production (10). The release of granule proteases is important for neutrophil migration and is partially responsible for their microbicidal activity (11, 12). Our group demonstrated previously that coculture of necrotic neutrophils and L. amazonensis–infected macrophages induced killing of parasites through a mechanism dependent on TNF-α and neutrophil elastase (NE) (13).

In this article, we show that coculture of neutrophils activated with fibronectin (FN), but not other ECMs, and L. amazonensis–infected macrophages reduces the parasite burden. This interaction leads to
the release of neutrophil granule proteins recognized by TLRs, which triggers parasite killing by infected macrophages.

**Materials and Methods**

**Ethics statement**

This study was approved by the Institutional Review Board of Human Ethical Research Committee of Fundação Oswaldo Cruz-Bahia, under number 100/2006.

**Neutrophil and macrophage culture**

Human blood was obtained from healthy volunteers from HemoCentro do Estado da Bahia (Bahia, Brazil). Human neutrophils were isolated by centrifugation using PMN medium, according to the manufacturer’s instructions (Axis-Shield, Dundee, Scotland). PBMCs were isolated by Ficoll-Hypaque gradients, plated in a 24-well tissue culture plate (Corning, Costar, NY) containing glass coverslips, and incubated at 37°C and 5% CO₂ for 30 min. Adherent cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM/ml 1-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin for 7 d.

**Leishmania culture and macrophage infection**

*L. amazonensis* (MHOM/BR/87/BA125) promastigotes were cultured at 23°C in Schneider medium, supplemented with 10% FBS, 2 mM/ml 1-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Macrophages were cultured on glass coverslips for 4 h with *L. amazonensis* in the stationary phase, at a parasite/cell ratio of 1:2. Wells were washed to remove extracellular parasites.

**Neutrophil-ECM activation assay**

Neutrophil activation was induced by ECM, including 10 μg/ml FN, 300 μg/ml collagen type I (CL), and 20 μg/ml laminin (LM) (all from Sigma-Aldrich, St. Louis, MO). Sterile 96-well tissue culture plates were coated with ECM proteins diluted in RPMI 1640 (14). After protein coating, 5 × 10⁶ neutrophils in RPMI 1640 supplemented medium were added for 1 h at 37°C and 5% CO₂. Following this incubation, treated neutrophils were harvested, counted, and verified for their activated profile by the production of inflammatory mediators, degranulation, and electron microscopy.

**Coculture of *L. amazonensis*-infected macrophage with activated neutrophils**

After the infection of macrophages, FN-activated neutrophils were added to the cultures at a neutrophil/macrophage ratio of 5:1 in complete RPMI 1640 medium. Cocultures were performed on glass coverslips; cells were harvested after 24 h of incubation, fixed with methanol, and stained with H&E. Results are shown as parasite number/100 macrophages (parasite burden) and as a percentage of infected macrophages. In some experiments, cultures were performed in the presence of the following Abs or pharmacological inhibitors: anti-human NE (50 μg/ml), anti-human TNF-α (6 μg/ml), anti-IgG mouse control (6 μg/ml), anti-IgG rabbit control (50 μg/ml), myeloperoxidase (MPO) inhibitor (benzoic acid hydrazide analog, 0.1 μg/ml), TIMP (matrix metalloproteinase 9 [MMP-9] inhibitor, 30 ng/ml), zileuton (10 μg/ml), and anti-TLR4 (1 μg/ml). MMP-9 (40 ng/ml), fMLF (50 nM) as a positive control, or DMSO as a vehicle control for cell activation. Subsequently, neutrophil degranulation was measured by MMP-9 release and NE activity in culture supernatants. Exposure of neutrophils to FN induced greater release of MMP-9 (Fig. 1A) and NE activity (Fig. 1B) compared with resting cells or other proteins.

To confirm this activated profile induced by FN, the ultrastructure of cells and ROS production were evaluated. Resting neutrophils present their regular shape, cytosolic granules with electron-dense appearance (red arrow), and multilobulated nucleus (Fig. 2A). In contrast, when neutrophils are activated by FN (Fig. 2B), an accumulation of granules with a clear appearance close to the cell surface (red arrowheads) and plasma membrane projections (blue arrows) are observed, indicating cell degranulation (Fig. 2B). Additionally, greater ROS production was detected in FN-activated neutrophils (Fig. 2C). Together, these results demonstrated that FN induced the most efficient activation of neutrophils. Therefore, FN treatment was used in the remaining experiments to induce neutrophil activation.

Finally, zymosan particles (five particles to one neutrophil) were added to cultures of FN-activated or resting neutrophils for 1 h. The aim of this assay is to determine whether neutrophil activation alters its phagocytosis ability. No differences were observed in zymosan phagocytosis between the groups (data not shown).

**Results**

**Treatment of human neutrophils with FN in vitro induces degranulation and ROS production**

Neutrophil granules store proteins that are critically important for neutrophil endothelial transmigration and inflammatory responses (11, 17, 18). To mimic neutrophil activation by transmigration, resting neutrophils were exposed to the following immobilized ECM proteins (14) for 1 h: FN, CL, LM, and fMLF, as a positive control for cell activation. Subsequently, neutrophil degranulation was measured by MMP-9 release and NE activity in culture supernatants. Exposure of neutrophils to FN induced greater release of MMP-9 (Fig. 1A) and NE activity (Fig. 1B) compared with resting cells or other proteins.

Samples were incubated at 37°C for 3 d. NE activity was measured by reading absorbance at 410 nm. Intracellular reactive oxygen species (ROS) levels were measured by staining with the oxidative fluorescent dye probe dihydroethidium (10 μM; Invitrogen) for 30 min at 37°C and analyzed by flow cytometry. Data are displayed as graphs produced using FlowJo software (TreeStar). Supernatants from *L. amazonensis*-infected macrophages cultured alone or cocultured with activated neutrophils were collected and assayed for TNF-α by ELISA (BD Bioscience, San Diego, CA), MMP-9 by colorimetric test (R&D Systems), and LTB₄ by an enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MD).

**Transmission electron microscopy**

After FN treatment, cells were centrifuged, and pellets were resuspended and fixed with 1% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. Cells were washed using the 0.1 M phosphate buffer and embedded in molten 2% agar (Merck). Agar pellets containing cells were postfixed in a mixture of 1% phosphate-buffered osmium tetroxide and 1.5% potassium ferrocyanide for 1 h and processed for resin embedding (PolyBed 812, Polysciences). The sections were mounted on uncoated 200-mesh copper grids and viewed using a transmission electron microscope (JEM-1230).

**Statistical analysis**

Comparisons between two groups were performed using the Mann–Whitney test, whereas the Kruskal–Wallis test, followed by the Dunn test, was used for multiple groups. All tests were performed using GraphPad Prism 5.0 (GraphPad, San Diego, CA). Differences with p < 0.05 were considered significant.

**Discussion**

Our group already showed that interactions between apoptotic neutrophils, but not necrotic ones, increased parasite burden in human macrophages infected with *L. amazonensis* (13). However, the consequences of the interaction between infected macrophages and activated neutrophils are not known. Therefore, we cocultured...
FN-activated neutrophils and *L. amazonensis*-infected macrophages (5:1 ratio). After 24 h, a significant decrease in the percentage of infected macrophages (Fig. 3A) and parasite burden (Fig. 3B) was observed with the presence of FN-activated neutrophils compared with infected macrophages alone or those cultured with resting neutrophils. Different time points were also evaluated (i.e., 1, 48, and 72 h). After 1 h of interaction, ~25% of macrophages were infected, and the presence of FN-activated neutrophils gradually reduced the percentage of infected macrophages and parasite burden over time (data not shown). Therefore, the time point of 24 h was chosen for the remaining experiments.

FN-activated neutrophils were also cultured separately from infected macrophages using TWs. The reduction in the percentage of infection (Fig. 3C) and in the parasite burden (Fig. 3D) of macrophages was maintained in TW cocultures. These results suggest that the reduction in the infection on macrophages is independent of cell contact and may be mediated by soluble mediators.

**Enzymes from neutrophil granules participate in the killing of *L. amazonensis* by macrophages**

Exocytosis of cytoplasmic granules plays an important role in the regulation of many neutrophil functions (19), including the control of *Leishmania* infection (6). Next, the activity of NE and MPO, as well as MMP-9 production, was assayed in the supernatants of 24-h cocultures from FN-activated neutrophils and *L. amazonensis*-infected macrophages. This resulted in a higher activity of NE compared with infected macrophages alone (Fig. 4A). To verify whether NE was involved in parasite killing, coculture was...
performed in the presence of anti-human NE-neutralizing Ab. The addition of anti-NE Ab (Fig. 4B), but not an IgG-isotype control (data not shown), restored the parasite burden observed in the absence of FN-activated neutrophils (Fig. 4B).

MPO is involved in the oxidative response of neutrophils by inducing the generation of hypochlorous acid, which is a potent microbicidal product (20). There is a significant increase in MPO activity in the supernatant from cocultures (Fig. 4C). The addition of MPO inhibitor reverted the leishmanicidal effect of infected macrophages in the presence of FN-activated neutrophils (Fig. 4D).

Finally, MMP-9 is an important enzyme for neutrophil migration because it degrades the ECM. Furthermore, products released by this process seem to be responsible for the inflammatory response (19, 21). As shown in Fig. 4E, there is a significant increase in MMP-9 production by cocultures (Fig. 4E), and its inhibition by TIMP, a natural MMP-9 inhibitor (22), restored the parasite burden observed in the cultures of macrophages alone (Fig. 4F). Taken together, these results suggest the involvement of neutrophil granule release, induced by FN activation, in the parasite killing of L. amazonensis–infected macrophages.

Proinflammatory mediators are involved in the leishmanicidal activity of macrophages

We next investigated whether inflammatory mediators, such as TNF-α and LTB₄, could also be involved in parasite killing. TNF-α concentration was measured in fresh supernatants after 4 h of interaction between FN-activated neutrophils and L. amazonensis–infected macrophages. The results showed a higher level of TNF-α in the presence of FN-activated neutrophils (Fig. 5A); the addition of anti–TNF-α Ab to the coculture increased the parasite burden in infected macrophages (Fig. 5B). No differences were observed when isotype-control Ab was added to the coculture (data not shown). These results suggest the participation of this cytokine in the control of L. amazonensis infection by macrophages.

LTB₄ is a proinflammatory lipid mediator produced from arachidonic acid metabolism by 5-LO and is involved in the increase in phagocytic ability, oxidative response, and killing of pathogens (6, 23, 24). Next, we investigated whether LTB₄ is involved in the leishmanicidal ability of infected macrophages cocultured with FN-activated neutrophils. The results showed a higher level of LTB₄ in the cocultures compared with L. amazonensis–infected macrophages alone (Fig. 5C). Next, cocultures were treated with Zileuton, a 5-LO inhibitor, and the parasite burden of infected macrophages was significantly increased (Fig. 5D). This suggests that LTB₄ production is also involved in the killing of L. amazonensis by macrophages.

To verify the source of TNF-α and LTB₄, these inflammatory mediators were quantified in the supernatants of isolated FN-activated neutrophils. FN-activated neutrophils failed to produce TNF-α or LTB₄; only in the cocultures with L. amazonensis–infected macrophages was it possible to detect these mediators (data not shown). Killing of L. amazonensis is mediated by LTB₄ produced by macrophages after neutrophil degranulation

LTB₄ and TNF-α derived from the coculture are crucial for the control of L. amazonensis infection (Fig. 5). In this way, we
sought to investigate the role of neutrophil degranulation by treating cells with WT, an exocytose inhibitor (25). The results indicated that inhibition of degranulation by WT (WT/FN) reverted the reduction in the percentage of infection (Fig. 6A) and parasite burden (Fig. 6B) induced by the presence of FN-activated neutrophils.

Next, we sought to test whether LTB4 production is triggered directly by neutrophil degranulation. The presence of FN-activated neutrophils increased LTB4 production in the coculture with L. amazonensis macrophages (Figs. 5C, 6C). The inhibition of specific granule enzymes, such as MPO, MMP-9, and NE, reduced LTB4 production (Fig. 6C). However, when neutrophil degranulation was inhibited, LTB4 production was completely abrogated (Fig. 6C). Similar results were observed for TNF-α production under these conditions (data not shown).

Taken together, these results suggest that neutrophil degranulation induces the activation of macrophages, which produces LTB4 and TNF-α, responsible for parasite killing and control of the infection.

**Neutrophil granule enzymes are recognized by macrophages through TLRs**

The ability of neutrophil degranulation or enzymes from neutrophil granules, such as NE, MPO, or MMP-9, to induce the production of TNF-α and LTB4 (Fig. 6) suggests that these enzymes interact with membrane-associated recognition receptors that signal cytokine expression. Although other pattern-recognition receptors could induce cytokine expression, the implications of TLR2 and TLR4 activation are largely described as leading to this effect (6, 26–28). In this way, we assessed the response of infected macrophages in the absence of TLR signaling. In order to achieve that, infected macrophages were treated with neutralizing Abs against TLR2 and TLR4 simultaneously to block these signaling pathways. Then, purified enzymes (MPO, NE, and MMP-9) were added simultaneously to the culture to mimic neutrophil degranulation. In the presence of neutrophil enzymes, the infection of macrophages is reduced (Fig. 7A), and the production of LTB4 is increased (Fig. 7B). These effects are no longer observed when TLR2 and TLR4 signaling pathways are neutralized (Fig. 7).

Together, these findings show that enzymes derived from neutrophil degranulation are recognized by TLRs on macrophages. This induces the production of LTB4 and TNF-α, leading to parasite killing and inflammation.

**Discussion**

The cross-talk between neutrophils and macrophages is critical to the outcome of infection by *Leishmania* parasites. In this article, we expand the knowledge on the interaction between neutrophils and macrophages, mimicking the features at the inflammatory site, where neutrophils arrive activated after migration through the endothelium. We show that the interaction between neutrophils activated by FN and *L. amazonensis*-infected macrophages also decreases the infection rate. This leishmanicidal effect was dependent on the release of neutrophil granule enzymes, such as NE, MPO, and MMP-9, as well as on the production of inflammatory mediators TNF-α and LTB4 through TLR signaling.

The presence of granule enzymes in the lesion, where infected macrophages reside, depends on the recruitment of neutrophils from the blood stream. Neutrophil migration to the inflammatory site involves several controlled events, such as adhesion to endothelial and extracellular matrix proteins (29, 30). This process provides neutrophils with a functional response that can be amplified upon exposure to another stimulus. In the current study, FN induced efficient neutrophil priming compared with other ECM proteins. It was shown that LM and CL are constitutively present in the ECM (31). In contrast, FN circulates in the soluble form in plasma or accumulates in tissue as insoluble ECM components (32). There is a marked upregulation of FN in the plasma during inflammatory conditions (33, 34), and there is extravasation of this plasmatic protein in the basement membrane of the inflamed tissue (35). Moreover, FN has the ability to act as an endogenous ligand for TLR4 and to activate its signaling pathway, which leads...
to the activation of NF-κB. This TLR4-agonist activity contributes to disease progression and stimulates the inflammatory cascade (36, 37).

However, full neutrophil activation is a two-step process, beginning with priming by an initial exposure to priming agents, such as cytokines and ECM proteins. The second stimulus is the activating agent or agonist, which enables the neutrophils to perform their microbicidal and proinflammatory functions (38). In agreement with that, addition of FN-activated neutrophils to the culture of infected macrophages enhanced parasite killing. Based on our findings from the TW experiments, this second stimulus can occur without cell contact. In fact, we observed that purified enzymes from neutrophils also stimulate macrophages through TLR2 and TLR4, leading to LTB4 production and, subsequently, the killing of parasites.

This study also identified a novel and significant role for FN in neutrophil priming: the production of LTB4 from infected macrophages. Priming of neutrophils with FN or *L. amazonensis* infection of macrophages did not induce LTB4 production. However, when these cells are cocultured, it is possible to detect it in the supernatant. Based on our findings from the TW experiments, this second stimulus can occur without cell contact. In fact, we observed that purified enzymes from neutrophils also stimulate macrophages through TLR2 and TLR4, leading to LTB4 production and, subsequently, the killing of parasites.

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Leukotrienes are eicosanoids derived from the 5-LO metabolism of arachidonic acid to form LTB4 and cysteinyl leukotrienes. LTB4 acts on G protein-coupled receptors BLT1 and BLT2. This signaling results in increased intracellular calcium, which activates protein kinases, culminating in different cellular and tissue responses, including TNF production (8, 39, 40). It was shown that *L. amazonensis* infection did not alter LTB4 production by murine macrophages; however, ATP stimulation was able to induce LTB4 production (41), and this could lead to TNF-α release (40, 42), although infected macrophages had decreased LTB4 release compared with uninfected cells exposed to ATP. These findings reinforce the ability of *L. amazonensis* to downmodulate LTB4 production by macrophages. Data previously published have shown that it may be possible to reverse these effects by adding exogenous ATP or neutrophil enzymes. In contrast, the infection of human macrophages with *L. braziliensis* induces LTB4 production after 30 min (43); however, *L. braziliensis* infection downregulates BLT1 expression, probably to subvert the leishmanicidal activity induced by LTB4. Based on these findings, *Leishmania* parasites developed different mechanisms to evade the immune response of macrophages, but the induction of LTB4 after specific stimulation opens the possibility to revert this downmodulation, leading to parasite killing. In this way, we confirmed that *L. amazonensis*-infected macrophages do not produce LTB4 or TNF-α; however, ATP, stimulation of TLR signaling by neutrophil enzymes is able to revert this effect, leading to the control of infection.

It is well known that *Leishmania* growth and persistence are associated with the subversion of macrophage immune response (41, 44–46). However, it was demonstrated that is possible to
revert this modulation. Neutrophils could act by providing potent antimicrobial molecules to the macrophage. This mechanism was initially proposed in a mouse study of experimental mycobacteriosis (47); later, this mechanism was also observed in infection by *Legionella pneumophila* (48). Neutrophil granule enzymes, such as NE, could activate the microbicidal functions of macrophages through interactions with surface receptors (49). TLR2 and TLR4 could be trigged by proteolytically active NE on infected macrophages, leading to an oxidative response, TNF-α production, and parasite killing (26, 50–52). Our data indicate that each en-

![FIGURE 6.](image)

**FIGURE 6.** Products from neutrophil degranulation mediate *L. amazonensis* killing by infected macrophages through LTB₄. Neutrophils were treated or not with WT to inhibit degranulation and, after FN activation, were cocultured with infected macrophages. The monolayers were stained with H&E, and the percentage of infection (A) and parasite burden (B) were assessed after 24 h. (C) Additionally, supernatants from cocultures treated with different inhibitors were collected 24 h later and assayed for LTB₄ production. Data are shown as the median from five donors (horizontal line), and statistical analysis was performed using Mann–Whitney *U* test. *p < 0.05, **p < 0.01. FN-PMN, FN-activated neutrophils; IM, *L. amazonensis*-infected macrophages; M₀, macrophages alone; α-MPO, anti-MPO Ab; α-NE, anti-NE Ab; TIMP, MMP-9 inhibitor.

![FIGURE 7.](image)

**FIGURE 7.** Enzymes from neutrophil degranulation activate macrophages to kill *L. amazonensis* and to produce LTB₄ through TLRs. Infected macrophages were treated with purified enzymes (NE, MPO, and MMP-9 [Enzymes]) for 24 h or pretreated for 1 h with neutralizing Abs against TLR2 and TLR4 (αTLRs/Enzymes) or isotype as control (Isot/Enzymes). Subsequently, cells were stained with H&E to evaluate the infection index (A), and LTB₄ production was measured in the supernatant (B). Values from control group were set as one and are shown as the median from five donors (horizontal line). The statistical analysis was performed using Mann–Whitney *U* test. *p < 0.05, **p < 0.01, ***p < 0.001.
zyme has a role in the activation of macrophages, because their individual inhibition reduced LTB4 production. However, when the entire degranulation process is inhibited, the production of LTB4 is abrogated, suggesting that the enzymes act in synergy. In summary, our results indicate that products from neutrophil degranulation, such as MPO or NE, could be recognized by infected macrophages, leading to their activation and the killing of *L. amazonensis* through LTB4 and TNF production. It was shown that the effects of TNF-α on ROS production are dependent on 5-LO (53). We could not exclude the possibility that FN-activated neutrophils, in the presence of *L. amazonensis*-infected macrophages, are able to release these mediators. Additionally, our group recently demonstrated that *L. amazonensis* infection of human neutrophils in vitro induced ROS production in an LTB4-dependent fashion (6).

Our results suggest that the recruitment of neutrophils could be implicated in the inflammatory response observed on the lesion caused by Leishmania. In this way, the neutrophils could contribute to the reduction in the number of parasites. At the same time, activated neutrophils would promote local inflammation. This study also contributes to the understanding of the mechanisms involved in the innate immune response against *Leishmania*.

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**Disclosures**

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